THIS NUMBER COMPLETES VOLUME 37

VOLUME 37 NUMBER 6

Medical Library

JOURNAL OF BACTERIOLOGY

OFFICIAL ORGAN OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

JUNE, 1939

EDITOR-IN-CHIEF
C.-E. A. WINSLOW

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Published Monthly by THE WILLIAMS & WILKINS COMPANY
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Entered as second-class matter April 17, 1916 at the postoffice at Baltimore, Maryland, under the Act of March 3, 1879.
Subscription price $5.00 per volume. Single numbers, $1.00.

EVALUATION OF GERMICIDES BY A MANOMETRIC METHOD[1]

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Received for publication October 25, 1938

The efficacy of germicides as chemotherapeutic agents is determined by a variety of factors, among which the relation between toxicity of a compound for the tissues of the host and for the parasite respectively is of greatest importance. In order to be useful, the disinfectant should possess the highest possible germicidal efficiency, and the least possible toxicity for animal cells. The ratio between these two properties constitutes a presumptive measure of its efficacy. It is of obvious importance to possess experimental methods for the measurement of this ratio, to serve as a guide to clinical investigation, which alone can establish the actual therapeutic value of germicidal agents.

The bactericidal efficiency of a disinfectant is usually determined by one of several procedures, which consist essentially in finding the concentration of the substance in question which will sterilize a suspension of bacteria in a definite period of time under carefully controlled conditions of exposure. The result is often expressed in terms of the ratio between the effective concentration of the disinfectant under investigation and that of phenol or mercuric chloride. The coefficient obtained by this procedure is sometimes supplemented by the result of a similar test carried out in the presence of added organic matter. As commonly determined, the phenol coefficient serves as a convenient if somewhat arbitrary measure of the capacity of a disinfectant to destroy the bacteria present in non-living substrata.

[1] Aided by a grant from the Blue Line Chemical Company of St. Louis, Missouri.

In order to determine the toxicity of disinfectants, the common practice consists in finding the minimal lethal dose for a given experimental animal following subcutaneous, intraperitoneal or intravenous injection. The results of these tests may then be combined into a single value (toxicity index), expressed as the ratio of the minimal concentration effective against bacteria to the M.L.D. for animals. In this procedure the assumption is made that compounds giving favorable results in the treatment of infectious diseases will be found among disinfectants exhibiting low indices of toxicity.

It is evident that the search for compounds possessing selective action of this kind will be promoted by the availability of simple and rapid methods of evaluation. Toxicity measurements requiring the observation of animals is cumbersome, time-consuming, and inherently difficult of control. The measurement of phenol coefficients is also subject to variations inherent in the methods. Sufficient evidence of this is seen in the fact that the copious literature on disinfectants contains many conflicting data. To escape some of these difficulties, the measurement of toxicity by the use of tissue cells in culture has been suggested by Lambert (1916), Lambert and Meyer (1926), and used by German (1928), Birkhaug (1930), and Buchsbaum and Bloom (1931). Recently Salle, McOmie and Schechmeister (1937–1938) have proposed a systematic method based on toxicity measurements of this kind. Their procedure is, however, laborious, and retains the limitations of the standard germicidal test. It occurred to us that simple and relatively rapid evaluation of toxic effects upon tissues might be secured by observing the effect of disinfectants on the consumption of oxygen by tissue cells. Such a procedure promised the advantages of other *in vitro* methods in permitting the application of the same criteria to the measurement both of toxicity and of germicidal action, and in employing similar experimental conditions meanwhile. It was found, as anticipated, that tests on animal cells and on bacteria yielded reproducible results relatively independent of the minor variations in technique which affect the outcome of the conventional qualitative tests so greatly (Bronfenbrenner,

Hershey and Doubly, 1938). It remained, however, to establish the relationship between the effect of disinfectants on respiratory activity, and on viability. Comparative studies directed to this end are reported in the present paper.

EXPERIMENTAL

The methods followed in comparing the effect of disinfectants on the oxygen consumption of mouse liver, and of *Escherichia coli* respectively, have already been described in brief (Bronfenbrenner, Hershey and Doubly, 1938). They consisted essentially in determining the concentration of disinfectant in the test fluid necessary to cause 50 per cent reduction in the rate of oxygen uptake, measured between the 15th and 20th minutes after the beginning of the experiment in either case. The same procedure has now been applied to *Staphylococcus aureus*. These tests have been carried out with a few representative antiseptics, and the results have been compared with those given by the usual methods for the measurement of toxicity and germicidal values.

The manometric measurement of toxicity

One gram of fresh mouse liver was triturated in a mortar with 20 ml. of 0.85 per cent saline. Into each vessel of the respirometer were placed 2 ml. of the resulting liver cell suspension, 1 ml. M/10 phosphate buffer of pH 7.4, 1 ml. 5 per cent sodium succinate and, in the side arm, 1 ml. of aqueous solution of the disinfectant in five times the concentration to be tested. The vessels were attached to Barcroft differential manometers, and allowed to remain 20 minutes in the water bath at 37 $\pm$0.001 °C. At the end of this time the disinfectant was tipped into the cell suspension, and the rate of oxygen consumption for successive five-minute intervals was recorded during thirty to forty minutes. An example of the type of data obtained has been illustrated in figure 1. The difference between the rate of oxygen uptake observed during the 15th to the 20th minute after addition of the disinfectant, and that of the corresponding rate for the control (water substituted for disinfectant) was expressed as a per cent of the latter. These values were plotted against the respective

concentrations of the disinfectant tested, and from the curve obtained (see fig. 2), the concentration corresponding to 50 per cent inhibition could be interpolated. This arbitrary end-point has been chosen for comparative purposes only, largely because the accuracy and reproducibility of the measurements are greatest in this region, as inspection of the curves reveals.

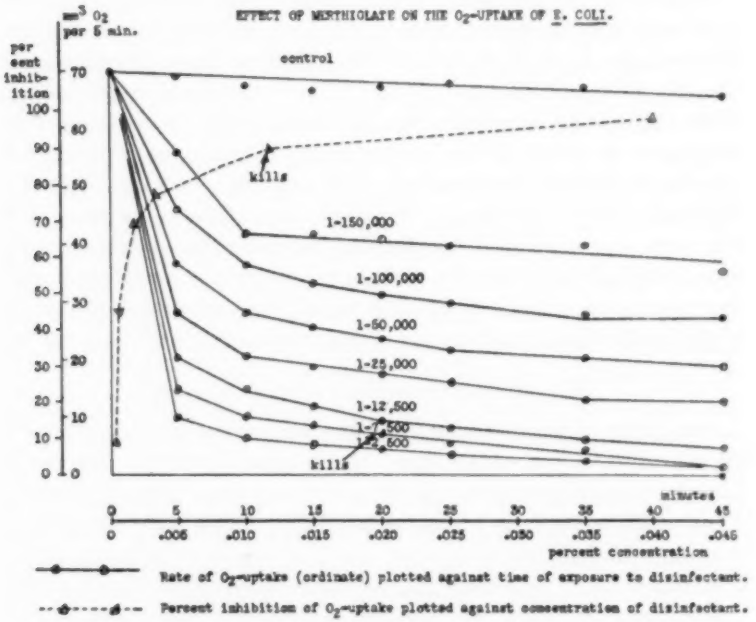

FIG. 1

The manometric measurement of antiseptic action

To facilitate measurement, the number of bacteria employed in the test was adjusted to give a rate of oxygen consumption closely approximating that obtained with the tissue cells. This required approximately 3×10^9 organisms in the case of *E. coli* in glucose buffer. This quantity of bacteria corresponds to 0.09 mgm. of bacterial nitrogen as compared with 3.2 mgm. in 0.1 gram of liver cells. In order to approximate the conditions

in the two tests, the difference in amount of organic matter present was roughly made up by addition to the bacterial sus-

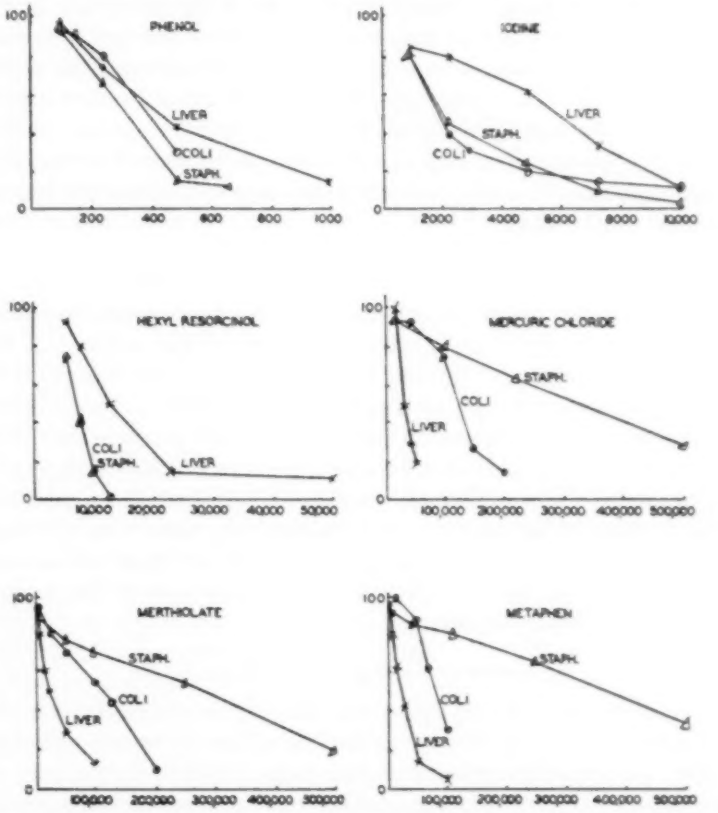

FIG. 2. EFFECT OF ANTISEPTICS ON THE OXYGEN UPTAKE OF E. COLI, STAPH. AUREUS, AND MOUSE LIVER CELLS

The per cent inhibition of O_2-uptake (ordinates) is plotted against the dilution of the disinfectants (abscissae).

pension in the respirometer of 0.2 ml. of horse serum (2.2 mgm. nitrogen).[2] The test mixture in each vessel contained approxi-

[2] It is, of course, desirable to carry out these tests in the presence of additional organic matter, also in order to approach more nearly the *in vivo* conditions.

mately 3×10^9 viable organisms obtained from the centrifugate of 24-hour-broth cultures and suspended in 1.8 ml. of saline, together with 0.2 ml. of horse serum, 1 ml. M/10 phosphate buffer of pH 7.4, and 1 ml. of 10 per cent glucose. The test was conducted, and the results were expressed exactly as described above for the liver cells. The experiments with *Staphylococus aureus* followed the same procedure, but it was found desirable to suspend the organisms in supernatant from the broth cultures, rather than in saline, in order to obtain a constant rate of oxygen uptake in the control flask.

Direct measurement of bactericidal value

In order to judge the efficiency of the manometric method, parallel bactericidal tests were conducted by the method of the F. D. A. (Ruehle and Brewer, 1931) described by Reddish (1927), except that *E. coli* was used as the test organism. To make the comparison more exact, we have carried out at the same time modified tests in which the numbers of bacteria, time of exposure, and composition of the medium were so adjusted as to duplicate the conditions obtaining in the manometric tests. In addition, similar tests were performed in which each of these factors was varied independently. Only the more pertinent of these data will be presented at this time.[3]

The measurement of bacteriostatic action

Anticipating that not only the numbers of bacteria actually destroyed, but also the bacteriostatic effect of lower concentrations of the disinfectants tested might influence the rate of oxygen uptake, we included bacteriostatic tests for comparative purposes.

Various dilutions of each of the disinfectants were made in

[3] The strain of *E. coli* used here was killed at 37°C. under the standard conditions (Reddish, 1927) by 1–120 dilution of phenol, and gave the following phenol coefficients: mercuric chloride 458, merconyl 333, metaphen 227, merthiolate 68, iodine (tincture U.S.P.) 62, hexyl resorcinol 45. The strain of *S. aureus* used (freshly isolated from human lesion) was killed under these conditions by 1–95 dilution of phenol, and gave with the same disinfectants the following coefficients: 79, 87, 66, 52, 66, 79.

broth, and 10 ml. of each dilution were seeded with a 4-mm. loop from a 1-10 dilution of a 24-hour broth culture of *E. coli* and *S. aureus* respectively. The highest dilution in which growth was inhibited during 48 hours incubation at 37°C. was taken as the bacteriostatic titre.

The measurement of toxicity in vivo

In order to correlate the results of the manometric tests on liver cells with toxicity to the living animal, the minimal lethal dose of each disinfectant was determined by injecting intraperitoneally 1 ml. amounts of various dilutions into mice weighing 22 to 26 grams. The minimal dose in grams per kilo regularly killing the animals within 48 hours was taken to be the M.L.D.

DISCUSSION OF RESULTS

The effect of the disinfectants on oxygen uptake of both liver cells and bacteria was manifested with surprising abruptness, as compared with the rate of death, for example, of the bacteria. No marked difference in kinetics of this effect was seen with the three types of cells, or with the various concentrations of the several disinfectants used, except that the action of the mercurial compounds was slower in general than that of the other disinfectants tested. The data given in figure 1 are, therefore, illustrative of the effects seen throughout. There, it is shown that the most marked depression in the rate of respiration occurred almost instantaneously, so that beyond the first 5 or 10 minutes the per cent of inhibition as compared with the control remains almost unchanged throughout the period of observation. This is true irrespective of the initial concentration of disinfectants and of the extent of the initial drop. In other words, the per cent of inhibition of oxygen-uptake observed at any time after the first few minutes is clearly a function of the concentration of disinfectant (see fig. 1), but not of the duration of exposure. Why the disinfectant present in any one concentration should thus apparently cease to act soon after contact with the cells, while the latter remain exquisitely sensitive to increased concentrations of the disinfectant, is not clear. This circumstance

was, however, favorable to our purpose, since it virtually eliminated the time factor as one of the variables affecting the interpretation of results.

Of more immediate interest regarding the quantitative effect of the disinfectants on the respiration of bacteria, was to establish some correlation between bacteriostatic and bactericidal action on the one hand, and inhibition of oxygen-use on the other. Table 1 contains the significant data bearing on these relationships. Here the percentages of respiratory inhibition given in 20 minutes by the bacteriostatic and bactericidal dilutions,

TABLE 1

Per cent inhibition of O_2-uptake of E. coli and S. aureus given by bacteriostatic and bactericidal dilutions of several disinfectants

ANTISEPTIC	E. COLI				STAPH. AUREUS			
	Bactericidal dilution	Inhibition	Bacteriostatic dilution	Inhibition	Bactericidal dilution	Inhibition	Bacteriostatic dilution	Inhibition
		per cent		*per cent*		*per cent*		*per cent*
Mercuric chloride	50,000	91	700,000	10	3,000	95	250,000	65
Merconyl	50,000	90	250,000	10	7,500	95	250,000	60
Merthiolate	7,500	90	600,000	10	5,000	84	1,750,000	10
Metaphen	12,500	100	300,000	10	8,000	92	1,250,000	10
Hexyl resorcinol	<5,000	>77	10,000	15	<5,000	>77	25,000	5
Phenol	150	92	400	50	100	90	600	15
Iodine, tincture	1,000	82			800	90		

respectively, of the several disinfectants tested against *E. coli* and *S. aureus* are compared.

It may be seen that the maximal bacteriostatic dilutions of these substances usually depress the oxygen uptake by about 10 per cent, while effective germicidal dilutions do so in every case in excess of 80 per cent. The irregularity of the former values is probably due to the fact that bacteriostatic titres were necessarily obtained under conditions widely divergent with respect to numbers of bacteria, and the composition of the medium, from those employed in the manometric test. These factors would, of course, affect very largely the outcome of the bacteriostatic titration. The results with the dilutions exhibiting

bactericidal effects, which could be determined under conditions identical for the two tests, fall within a surprisingly narrow range. From this we conclude that in general the inhibition of oxygen uptake may be taken as a reasonably quantitative measure of germicidal action. The limitations of the method are shown in the extreme cases of iodine and metaphen tested against *E. coli*. The bactericidal dilution of the former inhibited the oxygen uptake of this organism by only 82 per cent, while that of metaphen completely suppressed oxygen uptake. It would appear that different disinfectants, perhaps affecting different vital mechanisms of the cell, may leave varying portions of the oxidative functions active after the organism is killed. In most cases, this residual oxygen uptake amounts to approximately 10 per cent of the original activity.

These findings suggest that in interpreting results of manometric tests in terms of germicidal action, better agreement might be obtained if ±90 rather than 50 per cent inhibition of respiration were adopted as the end point. Dilutions interpolated from the graphs (fig. 2) for both of these end points are shown in tables 2 and 3, those corresponding with 90 per cent inhibition manifestly falling closer to germicidal dilutions than those corresponding with 50 per cent inhibition. The latter values have been retained, however, since they can be determined more accurately, and since, as the tables show, phenol coefficients calculated from the respective values do not indicate any superiority of the one over the other for comparative purposes.

In general, both the dilutions giving 50 per cent inhibition of oxygen-use of bacteria and the relative efficiency calculated from these, fall between corresponding values obtained in the bacteriostatic and germicidal tests, respectively. Exceptions appear only in the case of mercuric chloride and merconyl, these compounds inhibiting respiration of staphylococci in higher dilutions than could be predicted from the bacteriostatic titrations. These discrepancies are again referable perhaps to the fact that the titration of growth-inhibiting activity was carried out in peptone-containing medium, which notoriously interferes with the action of these compounds in particular.

TABLE 2

Comparison of germicidal and manometric tests with E. coli

ANTISEPTIC	BACTERIOSTATIC TEST		GERMICIDAL TESTS				MANOMETRIC METHOD			
	Inhibition of growth in broth		Salle (1) serum and embryo extract *E. typhosa*		Conditions as in manometric test*		50 per cent inhibition		90 per cent inhibition	
	Effective dilution	Relative efficiency	Effective dilution	Relative efficiency	Effective dilution	Relative efficiency	Effective dilution	Relative efficiency	Effective dilution	Relative efficiency
Mercuric chloride	700,000	1,750.0			50,000	333.3	120,000	285.7	55,000	314.3
Merconyl	250,000	625.0			50,000	333.3	120,000	285.7	52,000	297.2
Merthiolate	600,000	1,500.0	2,660	14.3	7,500	50.0	110,000	261.9	7,500	42.9
Metaphen	300,000	750.0	5,665	30.4	12,500	83.3	85,000	202.4	42,000	240.0
Hexyl resorcinol	10,000	25.0	1,770	9.5	<5,000	<33.3	7,000	16.7	1,000‡	5.7‡
Phenol	400	1.0	186	1.0	150	1.0	420	1.0	175	1.0
Iodine, tincture			3,370†	18.1	1,000	6.7	2,350	5.6	750	4.3

* Killing 10^3 bacteria per ml. in 20 but not 15 minutes, 4 per cent serum.
† Lugol's solution.
‡ By extrapolation.
(1) Proc. Soc. E. B. M., 1938, **37**, 694.

TABLE 3

Comparison of results of germicidal and manometric tests with S. aureus

ANTISEPTIC	BACTERIOSTATIC TEST		GERMICIDAL TESTS				MANOMETRIC TESTS			
	Inhibition of growth in broth		Salle (1) serum and embryo extract		Conditions as in manometric tests*		50 per cent inhibition of O_2-uptake		90 per cent inhibition of O_2-uptake	
	Effective dilution	Relative efficiency	Effective dilution	Relative efficiency	Effective dilution	Relative efficiency	Effective dilution	Relative efficiency	Effective dilution	Relative efficiency
Mercuric chloride	250,000	417			3,000	30	360,000	1,143.0	24,000	240
Merconyl	250,000	417			7,500	75	350,000	1,111.0	38,000	380
Merthiolate	1,750,000	2,917	25	0.2	5,000	50	270,000	857.0	2,000	20
Metaphen	1,250,000	2,083	1,370	12.5	8,000	80	390,000	1,238.0	30,000	300
Hexyl resorcinol	25,000	41	1,620	14.7	5,000	50	7,000	22.2	1,000‡	10‡
Phenol	600	1.0	110	1.0	100	1.0	315	1.0	100	1.0
Iodine, tincture			3,520†	32.0	800	8.0	2,350	7.4	800	8.0

* Kills in 20 but not 15 minutes in presence of 4 per cent serum, etc.
† Lugol's solution.
‡ By extrapolation.
(1) Proc. Soc. E. B. M., 1938, **37**, 694.

The effective dilutions, and corresponding phenol coefficients, obtained in the manometric test using 90 per cent inhibition of oxygen-use as the end point, are in good agreement with the values determined *under similar conditions* by actual titration of bactericidal action. Notable exceptions occur in the test with *E. coli* only in the case of metaphen, the manometric coefficient being about three times higher. With *S. aureus*, three of the four mercurials tested showed this peculiarity. Reviewing the evidence of tables 2 and 3, the conclusion seems warranted that the mano-

TABLE 4

Relative toxicity for mouse, mouse liver and chick heart

ANTISEPTIC	MOUSE		MOUSE LIVER (MANOMETRIC METHOD)				CHICK HEART (TISSUE CULTURE)	
	M.L.D. on intraperitoneal injection		50 per cent inhibition of O_2 uptake		90 per cent inhibition of O_2 uptake		Data of Salle*	
	Grams per kilo	Relative toxicity	Dilution	Relative toxicity	Dilution	Relative toxicity	Dilution	Relative toxicity
Mercuric chloride	0.0074	43.2	26,500	62.3	14,000	93.3		
Merconyl	0.0106	30.2	26,000	61.2	13,000	86.7		
Merthiolate	0.0435	7.3	18,000	42.3	2,400	16.0	4,220	18.8
Metaphen	0.0100	32.0	23,000	54.1	5,600	37.3	2,070	9.2
Hexyl resorcinol	0.0252	12.7	13,000	30.6	5,400	36.0	1,450	6.5
Phenol	0.3200	1.0	425	1.0	150	1.0	224	1.0
Iodine, U.S.P. tincture	0.0512	6.2	6,250	14.7	750	5.0	650	2.9

* Proc. Soc. E. B. M., 1938, **37**, 694.

metric phenol coefficient is an authentic index of the germicidal value of disinfectants, especially when it is not essential to distinguish between bacteriostatic and bactericidal action.

The same general conclusion may be drawn from table 4, giving results of toxicity tests conducted by the two methods. Specifically, the relative toxicity of the series for the surviving mouse liver parallels that found by *in vivo* test, the greatest discrepancies being encountered again in the case of the more bacteriostatic compounds. The reservation must be made, however, that intraperitoneal injection into the mouse does not give an absolute

measure of toxicity, the relative values obtained differing from those given when injection by another route is practiced, or when another experimental animal is used. Closer agreement between the manometric and *in vivo* tests could not, therefore, be anticipated. The results given in the protocol seem to justify the conclusion, as in the case of tests with bacteria, that the manometric method yields data possessing an intrinsic significance quite comparable with that given by any of the methods at present available. It is understood that the use of laboratory methods serves, at best, only for purposes of orientation preliminary to *in vivo* tests in experimental and natural infections. New compounds showing low indices of toxicity by the manometric method should deserve, *a priori*, especially intensive tests for therapeutic efficiency. The data presented, we believe, indicate that the simple and rapid method described can be depended on for the purpose of preliminary testing. It appears that the results obtained yield a measure of the combined effects of the disinfectant in destroying cells, and in inhibiting the activities of surviving cells. It may be taken as self-evident, that both toxicity and therapeutic properties of disinfectants are ultimately referable to some combination of lethal and inhibitive effects.

It is of interest to consider the antiseptic value of the series of disinfectants we have used. Figure 2 shows certain qualitative differences in an interesting way. By inspection of the curves, these disinfectants may be divided into two groups, the first including phenol, iodine, and hexyl resorcinol, by which liver cells are inhibited in much higher dilutions than bacteria; the second containing the mercurials of which the reverse is true. This difference is evaluated numerically in Table 5, showing toxicity indices of the various disinfectants calculated from manometric data, as well as from the germicidal and *in vivo* toxicity tests. Included also are indices from the data of Salle, McOmie and Schechmeister (1938) obtained by the tissue culture method. The standard methods (that is toxicity *in vivo*, and germicidal tests) with *E. coli* as the test organism, place the disinfectants examined in the following order of increasing toxic index: merconyl, mercuric chloride, merthiolate, iodine, metaphen, hexyl

resorcinol, phenol. The manometric method differs chiefly in placing iodine in sixth or seventh place. Salle's table for *Eberthella typhosa*, on the other hand, places iodine first, and merthiolate last. Using the staphylococcus as a test organism, the same disinfectants fall in the following series: merthiolate, hexyl resorcinol (?), merconyl, metaphen, iodine, phenol, mercuric chloride. The manometric method alters this series by moving mercuric chloride from last to third or fourth place, and hexyl resorcinol

TABLE 5

Toxicity indices obtained by various methods

ANTISEPTIC	B. COLI				B. AUREUS			
	In vivo in mouse*	Manometric method (mouse liver)†		Tissue culture method†	In vivo in mouse*	Manometric method†		Tissue culture method†
		50 per cent inhibition of O_2-uptake	90 per cent inhibition of O_2-uptake	Chick heart (Salle) (E. typhosa)		50 per cent inhibition of O_2-uptake	90 per cent inhibition of O_2-uptake	Chick heart (Salle)
Mercuric chloride	0.26	0.22	0.25		4.5	0.07	0.6	
Merconyl	0.19	0.22	0.25		1.2	0.07	0.3	
Merthiolate	0.30	0.16	0.32	1.6	0.46	0.07	1.2	169.0
Metaphen	0.80	0.27	0.13	0.4	1.2	0.06	0.2	1.5
Hexyl resorcinol	0.80	1.90	±5.40	0.8	0.8	1.9	±5.4	0.9
Phenol	2.1	1.00	0.86	1.2	3.1	1.3	1.5	2.0
Iodine	0.65	2.70	1.0	0.2‡	2.1	2.7	0.94	0.2‡

* Calculated by the formula $\frac{100}{\text{Germicidal Diln.} \times \text{MLD}}$.

† $\frac{\text{Dilution effective against tissue}}{\text{Dilution effective against bacteria}}$.

‡ = Lugol's solution.

from second to last. The method of Salle, as before, placed iodine first and merthiolate last.

The dependence of the result on the species of micro-organism used is illustrated in these data. No attempt has been made to determine the result of substituting other animal cells for those of the mouse liver, but the outcome would most probably vary with different tissues. The toxicity indices are, therefore, arbitrary in this sense, as they are in all methods. On the other

hand advantage might be taken of the greater flexibility of the manometric method in the study of infections of specific tissues.

The three methods compared in table 4 agree remarkably well in evaluation of toxicity. Discrepancies in the relative evaluation of these compounds are, therefore, primarily due to differences in results of the germicidal tests. These are most pronounced in the results with iodine and merthiolate. The findings of Salle and co-workers gave merthiolate a very poor rating, and led to the major conclusion that aqueous iodine was superior to any of the more complex germicides tested. Since this conclusion has a very important significance in connection with the trend of research on new disinfectants, particularly the organo-metallic compounds, it is of special interest that we have failed to confirm these results either by the standard methods or by the manometric method. It should be noted that Buchsbaum and Bloom (1931), using the tissue culture method, found, as we have, that iodine was inferior to some of the newer disinfectants.

SUMMARY

1. A manometric method for the measurement of germicidal and toxic action of disinfectants has been described which permits these properties to be evaluated in comparable terms. The method is simple and economical of time and materials.

2. Data for tissue toxicity obtained with several disinfectants are in satisfactory agreement with values obtained by intraperitoneal injection into the mouse.

3. Germicidal values against *Escherichia coli* in the manometric method compared favorably with the results of standard germicidal tests. With *Staphylococcus aureus* agreement was less close, but the divergence seemed to be well within the margin of error commonly met in germicidal tests.

4. The method would appear to be useful for preliminary evaluation of new disinfectants as an aid to research on their therapeutic value.

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THE SPECIFIC EFFECT OF ZINC AND OTHER HEAVY METALS ON GROWTH AND FUMARIC-ACID PRODUCTION BY RHIZOPUS[1]

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Received for publication November 19, 1938

Raulin (1869) first demonstrated that *Aspergillus niger* requires, besides the usual elements, traces of certain other elements, notably zinc, iron, silicon and possibly manganese. Although Raulin's claims have in some instances been refuted (Coupin, 1913; Lepierre, 1913; Wehmer, 1893), the investigations of Javillier (1907) and Bertrand (1911), and, more recently, of Steinberg (1919), Bortels (1927), and Roberg (1928) have confirmed and extended Raulin's original observations, especially with regard to the requirements for zinc and iron. It was found that if either of these two elements were removed completely from the medium, no growth would occur. Since the physiology of the fungus was modified in a unique fashion (Javillier 1912) by the presence of zinc, Javillier (1913) attributed to this element the rôle of a catalyst. Steinberg (1934) characterized zinc and iron as nutrients, as opposed to the stimulant concept of Pfeffer (1895). A detailed review of the literature on the heavy metal nutrition of fungi will be presented elsewhere (Foster 1939).

Few reports are found in the literature dealing with the relation of heavy metals to the growth of species of *Rhizopus*, most investigators previously having used *A. niger*. McHargue and Calfee (1931) found that the presence of zinc, especially when combined with manganese and copper, resulted in increases in *Rhizopus* growth. More recently zinc was found to play a vital rôle in the physiology of lactic-acid-producing strains of *Rhizopus* (Lockwood *et al.*, 1936; Waksman and Foster, 1938). Relatively

[1] Journal Series Paper, N. J. Agricultural Experiment Station, Department of Soil Microbiology.

poor growth occurred and large quantities of lactic acid accumulated in the medium unless zinc was added, when growth became greatly accelerated accompanied by sharp reduction in lactic acid yield. The zinc effect was interpreted (Waksman and Foster, 1938) as a catalyzation of the more complete destruction of the glucose thus rendering available more energy and carbon for the synthesis of cell substance.

EXPERIMENTAL

No special effort was made to free the medium completely of traces of heavy metals. The results, therefore, reflect the effect of the added zinc. Two hundred ml. portions of the basal medium containing Merck's reagent grade anhydrous glucose, 2.5 per cent; $(NH_4)_2SO_4$, 0.2 per cent; $MgSO_4 \cdot 7H_2O$, 0.05 per cent; K_2HPO_4, 0.05 per cent, were distributed into 500 ml. Erlenmeyer flasks. A strain of *Rhizopus nigricans*, previously found (Foster and Waksman, 1938) to produce fumaric acid from glucose vigorously and abundantly, was used in these investigations.

Zinc or other elements[2] were added to flasks in quadruplicate, which then were sterilized for 1 hour on 2 consecutive days at 100°C., and inoculated with spores from a zinc-free culture of *Rhizopus nigricans*. After incubation at 28°C. for 3 days, 5 gram portions of sterile $CaCO_3$ were added and the cultures shaken several times daily to neutralize the acid formed, care being taken not to injure the pellicle. After 7 and 11 days growth, the liquid was poured off aseptically and the pellicles washed twice with sterile distilled water which then was added to the original liquid. The pellicles were saved for later use. Glucose was determined by the Bertrand method, NH_3—N by distillation with MgO, calcium by $KMnO_4$ titration, after double precipitation as the oxalate; fumaric acid was determined gravimetrically as mercurous fumarate according to Ölander's (1929) modification of Hahn and Haarman's method. Since the results after 7 days are exactly comparable to the older set, only the latter are presented in table 1. All data are averages of duplicates.

Of the 5 elements tested, zinc exerted the greatest effect upon

[2] $ZnSO_4 \cdot 7H_2O$; $Fe_2(SO_4)_3$; Na_2MoO_4; $MnSO_4 \cdot 4H_2O$; $CuSO_4 \cdot 5H_2O$.

growth and acid production. Zinc ion, in a concentration of 1.2 parts per million, profoundly modified energy utilization by this organism, growth being decidedly stimulated. The organism grew vigorously and formed an abundant aerial mycelium over a

TABLE 1

Effect of trace elements upon growth and fumaric-acid production by Rhizopus nigricans

Milligrams per 200 ml. of culture, containing 5,088 mgm. glucose and 82.8 mgm. NH_3—N

ELEMENT	GLUCOSE CONSUMED	FUMARIC ACID FOUND	CONVERSION	NH_3—N CONSUMED	TOTAL CALCIUM IN SOLUTION	CALCIUM DUE TO ORGANIC ACIDS	PER CENT OF CALCIUM DUE TO FUMARIC ACID
mgm. per liter			*per cent*				
0	4,702	2,349	50.0	31.0	760.2	715.9	113.2*
5Zn	5,088	452	8.9	82.1	337.8	220.4	70.8
15Zn	5,088	439	8.6	81.0	332.5	216.7	70.0
5Fe	4,593	2,034	44.3	36.6	726.0	673.7	104.1
15Fe	4,227	1,878	44.4	25.4	652.8	616.5	104.4
5Mo	4,833	2,189	45.3	34.5	777.0	727.7	103.7
15Mo	4,873	2,114	43.4	36.6	770.3	718.0	101.5
5Mn	4,873	2,188	44.9	35.5	778.4	727.6	103.7
15Mn	4,812	2,009	41.8	36.0	763.8	712.6	97.2
2Cu	4,798	2,119	44.2	36.6	756.0	703.7	103.8
5Cu	4,862	1,643	33.8	47.4	653.3	585.6	96.2
5Zn + 5Fe	4,958	598	12.1	81.5	360.8	244.3	84.5
5Zn + 15Fe	4,863	597	12.2	82.2	352.5	235.0	77.4
5Zn + 5Fe + 5Mo	4,840	561	11.6	73.0	351.0	246.6	71.6
5Zn + 5Fe + 5Mo + 5Mn	4,968	377	7.6	82.2	289.5	172 0	75.6
5Zn + 5Fe + 5Mo + 5Mn + 5Cu	4,250	629	14.8	73.0	372.1	267.7	82.3

* Values greater than 100 per cent are due to fact that the acid was incompletely neutralized by the $CaCO_3$; later work showed this to be close to 100 per cent.

thick pellicle as contrasted to slow growth, very thin pellicle formation and lack of profuse aerial mycelium when zinc was deficient. The zinc-containing cultures made about two and a half times as much growth as the controls, as measured by nitrogen consumption.[3] The slight increase in glucose consumption

[3] The nitrogen content of *Rhizopus* cell substance was found to be approximately 5 per cent.

in the former cultures could in no way be compared to stimulation of growth. Zinc evoked even a more pronounced effect in the cultures which had additional supplementary trace elements (Fe, Mn, Mo, Cu). These elements alone had no significant effect, but the simultaneous presence of zinc brought about the characteristic response. One may thus speak of a "specific effect" of zinc on the growth of *Rhizopus*. Out of a large number of other so-called trace elements tested, none produced this effect.

The data on acid production (table 1) suggest the nature of this specific effect. In every case, a pronounced diminution in the actual amount of fumaric acid produced occurred in the zinc cultures. Despite the slightly greater utilization of glucose, the yields of acid which ordinarily approximated 44 per cent were reduced from $\frac{1}{3}$ to $\frac{1}{8}$ of this value by a trace of zinc. In the absence of this element, the organism transformed a large portion of the glucose into fumaric acid, whereas in its presence only a small fraction of the sugar consumed was left in the form of this acid. Zinc enabled the organism to utilize more completely its energy source instead of leaving a large part of it tied up in the form of fumaric acid. One may thus ascribe to zinc the rôle of a catalyst in the metabolism of *Rhizopus*. Mere traces seem to catalyze a more complete destruction of the glucose molecule and consequently its more efficient utilization as a source of energy and of carbon for cell synthesis. Zinc is not a mere accelerator of the rate of utilization of the original glucose as proven by comparing glucose-carbon disappearing with the carbon synthesized into cell substance.

$ZnSO_4$	GLUCOSE CARBON CONSUMED	CARBON SYNTHESIZED INTO CELL SUBSTANCE[1]	$\frac{\text{CELL SUBSTANCE C}}{\text{GLUCOSE C}}$
mgm. per liter	*mgm.*	*mgm.*	*per cent*
0	1,881	279	14.8
5	2,035	739	36.3
15	2,035	729	35.8

[1] This was calculated from the C:N ratio of the *Rhizopus* pellicle, which was taken as 9:1. Carbon content of *Rhizopus* was found by analysis to be approximately 45 per cent.

The efficiency of carbon utilization for cell synthesis was thus increased by 2 to 3 times under these conditions. Further evidence of the specific physiological effect of zinc upon the metabolism of *Rhizopus* was brought out by comparing the relation of fumaric acid to total acidity. When zinc was not added, fumaric acid accounted for practically 100 per cent of the total acidity measured as calcium in solution; in its presence this was only 70 to 80 per cent. These results have been repeatedly confirmed. In other words, zinc induced the formation of a very significant amount of other, as yet unidentified, acidic constituents.

In contrast to zinc, iron alone usually tended to depress growth but the fumaric-acid-yielding capacity of the organism was not reduced thereby (see also table 5). When combined with a trace of zinc, the effect of iron was not only completely obliterated, but the specific effect of zinc was even accentuated. The 3 elements (Mo, Mn, Cu) alone brought about no particularly specific response. Here again, regardless of what other elements were present in the medium, zinc favored the formation of another acid (or acids) amounting to 25 to 30 per cent of the total. Further study brought out that adjustment of the Fe:Zn ratio can reduce the specific effect of zinc and that the characteristic response of the organism to iron is manifested, i.e., a partial neutralization of the two elements takes place. It must be emphasized, therefore, that the associative or antagonistic nature of these two elements depends upon their ratio.

The marked response which zinc induces in *Rhizopus* suggests that the need for this element may be such that no growth would occur if it were completely removed from the medium. Fifty grams of $CaCO_3$ were added to two liters of the above medium which was then autoclaved for 15 minutes at 15 pounds pressure, according to Steinberg's (1919) purification technique and filtered hot. All glassware with which the medium came in contact had previously been thoroughly rinsed with distilled water purified in the same way. One milligram of the heavy metal salts was added to 100 ml. of medium in 250 ml. Erlenmeyer flasks, the media sterilized 1 hour at 100°C., and inoculated with a minimum of spores from a heavy-metal-deficient

culture. The same flasks had been used for previous experiments of a similar nature, each flask receiving identical metal treatment, so that contamination from the glass was at a minimum. After incubation at 28° for 4 days, the fungus pellicles were washed, dried at 80°C., over night and at 100°C. for 2 hours and weighed.

The scant few milligrams growth in the controls (table 2) is probably due to metals leaching from the glass as well as from the inoculum. The results clearly show that the combination of zinc and iron is indispensable for the growth of *Rhizopus*. Although there is some indication that manganese and copper may

TABLE 2

Growth of Rhizopus nigricans in purified basal nutrient solution as influenced by heavy metals

HEAVY METAL TREATMENT*	DUPLICATE DRY WEIGHT OF FUNGUS	SPORULATION
	mgm.	
Basal medium	11, 7	Sterile
Zn, Mn	25, 21	Sterile
Zn, Fe	264, 246	Very slight
Fe, Mn	10, 10	Sterile
Fe, Zn, Cu	318, 319	Abundant
Fe, Zn, Mn	325, 323	Abundant
Fe, Mn, Cu	12, 8	Sterile
Fe, Zn, Mn, Cu	355, 368	Abundant

* All metals except Cu were added as 10 mgm. of the following salts per liter of nutrient solution: $ZnSO_4 \cdot 7H_2O$, $Fe_2(SO_4)_3$, $MnSO_4 \cdot 4H_2O$; 2 mgm. per liter $CuSO_4 \cdot 5H_2O$.

also be essential, it is obvious that the amounts of these elements required by the organism are extremely low as compared with the demand for zinc and iron. The absence of either of the latter elements from the otherwise complete medium resulted in practically no growth, while the two elements combined always gave abundant growth.

The pellicles from the 7-day cultures of the first experiment were used to determine the influence of glucose concentration upon fumaric-acid production. Sterile glucose solutions (150 ml.) of varying concentrations were added to the flasks containing the pellicles together with 10 grams $CaCO_3$. Incubation was

no growth occurred, but the pellicles continued to convert the glucose into fumaric acid.

The results (table 3) show clearly that the rate of glucose consumption is influenced decidedly by its initial concentration. The maximum fumaric-acid production occurred when the initial concentration of glucose was from 5 to 15 per cent. Higher concentrations caused progressively smaller transformation of the sugar to acid. It is clear that efficiency of conversion is inversely proportional to glucose concentration. No consideration was given, in this experiment, to the differences in the size of the pellicles and to the possible effects of the various trace elements in the formation of the pellicles upon their activities. Many of the pellicles produced so much fumaric acid that abundant crystallization of calcium fumarate occurred in the cultures.

In the next experiment, a study was made of the effect of zinc, especially in combination with other elements, upon the specific activity of the pellicles produced. Pellicles were grown for 11 days, washed twice with sterile water, drained, 150 ml. sterile 5 per cent glucose solution and 5 grams sterile $CaCO_3$ added, and the flasks incubated 6 days at 28°C. with frequent shaking. The results presented in table 4 point to the fact that the history of the pellicle may alter the fumaric acid metabolism of *Rhizopus*. Pellicles grown with zinc alone and in combination with other elements were unfavorable to fumaric acid accumulation, whereas for glucose consumption their vigor was considerably reduced. A combination of Zn, Fe, Mo, and Cu in the original medium was most effective in repressing the production of acid by the fungus pellicles, both as regards percent conversion and total acids formed. In addition, considerable amounts of acids other than fumaric, as measured by the calcium in solution, were found among the products formed by the zinc pellicles. Further, the fact that fumaric acid accounted for only about 90 per cent of these acids, even in the case of the zinc-free pellicles shows that the pellicles metabolized the sugar differently from the growing cultures of the organism, the latter giving practically 100 per cent fumaric acid. Bernhauer (1927) reported similar behavior for citric-acid formation by mature pellicles of *Aspergillus niger*

TABLE 3

Influence of initial glucose concentration upon glucose consumption and fumaric-acid production by Rhizopus

150 ml. glucose solution used

INITIAL GLUCOSE CONCENTRATION	GLUCOSE CONSUMED	FUMARIC ACID FOUND	CONVERSION
per cent	*mgm.*	*mgm.*	*per cent*
2.5	3,323	1,559	46.9
5.0*	9,000	3,534	39.3
10.0	10,575	3,563	33.7
15.0	12,215	3,681	30.1
20.0	10,060	2,986	29.7
25.0	10,440	1,954	18.7
30.0	5,125	631	12.3
40.0	5,125	399	7.8

* These 2 flasks received 200 ml. of 5.0 per cent glucose solution.

TABLE 4

Influence of pellicle nutrition upon its effectiveness in producing fumaric acid

ELEMENTS IN ORIGINAL MEDIUM	GLUCOSE CONSUMED	FUMARIC ACID FOUND	CONVERSION	TOTAL CALCIUM IN SOLUTION	CALCIUM AS FUMARIC ACID
	mgm.	*mgm.*	*per cent*	*mgm.*	*per cent*
0	6,901	1,840	26.7	638.0	99.5
5Zn	3,235	1,010	31.2	392.0	88.5
15Zn	3,180	971	30.6	379.0	88.4
5Fe	6,820	2,879	42.2	1,048.0	94.9
15Fe	6,805	2,793	41.0	1,032.0	90.3
5Mo	7,157	2,681	37.5	938.0	98.6
15Mo	5,910	2,524	42.7	900.0	96.7
5Mn	6,820	2,395	35.1	841.0	98.2
15Mn	7,057	2,503	35.5	898.0	96.8
2Cu	7,057	2,409	34.1	853.0	97.4
5Cu	5,130	2,375	46.3	844.0	97.0
5Zn + 5Fe	1,987	319	16.1	131.0	83.9
5Zn + 15Fe	1,762	274	15.5	120.0	78.8
5Zn + 5Fe + 5Mo	2,125	319	14.1	136.0	80.9
5Zn + 5Fe + 5Mo + 5Mn	3,100	140	4.5	190.0	25.4
5Zn + 5Fe + 5Mo + 5Mn + 5Cu		344		190.0	81.3
10V	6,510	2,425	37.3	900.0	92.9

at 28°C., for 9 days, with frequent shaking. No nitrogen or nutrient minerals were present in these pellicle cultures. Thus,

cultivated in the presence of zinc and acting on sugar replacements.

A study was now made of the effect of zinc upon fumaric acid production, as influenced by the age of the fungus. Growth of the organism was interrupted at 3 different stages: (*a*) after 5 days, when the culture in the flasks with the regular medium had just formed a complete pad upon the surface of the medium and before the profuse aerial mycelium and sporulation occurred; (*b*) after 7 days, when a well-formed thick pad was produced, accompanied by heavy aerial mycelium and abundant sporulation, especially in the zinc cultures; (*c*) after 10 days, well past the maximum growth stage of the fungus growth. The experiment was set up in a manner similar to the previous, duplicate flasks of each treatment being withdrawn at each of the 3 periods of growth. The various pellicles were freed from the medium, washed, replaced as usual with 10 per cent glucose and $CaCO_3$ and incubated for 5 days. Besides zinc and iron alone, a combination of Fe, Mo and Cu was used, in order to demonstrate conclusively that it was zinc and not the other elements which resulted in the characteristic responses obtained in the previous experiment. Analyses of the culture liquid during the growth of the fungus are presented in table 5. The per cent conversion in the zinc cultures dropped from 22.6 to 12.8 per cent, with advancing age, thus tending to confirm definitely the assumption that the function of zinc becomes increasingly important in repressing fumaric acid production with increased age of the organism.

The earlier experiments showed that the formation of another unidentified acid is always associated with zinc. Further proof that this is correct is to be found in the fact that, with progressive age, when the effect of zinc became more marked, there was a progressive increase from 7.7 to 33.7 per cent in the quantity of the other acid found. Malic and succinic acids have been identified in culture solutions of this organism.

On comparing glucose and nitrogen consumption during the growth of the organism as influenced by iron, it is seen that this element repressed the latter. These cultures possessed a higher

sugar-fermenting capacity per unit of cell substance than the iron-free cultures. Both iron-containing and iron-free cultures, especially during the later periods of growth, had a fermentative capacity far greater than the zinc cultures. This is a highly important fact pertinent to the fundamental physiology of the organism, for the efficiency of its energy utilization thus becomes inversely proportional to its fermentative capacity. In other words, the zinc cultures consumed a small amount of glucose per unit of growth but utilized it very efficiently, whereas the zinc-free cultures consumed a large amount of glucose but very inefficiently.

Throughout the course of these investigations, the maximum conversion yields of fumaric acid were mostly from 40 to 46 per cent, yet, sporadically, yields were obtained as high as 58 per cent. In order to obtain a total increase from 36.5 to 45.1 per cent in the 5 and 7 day iron cultures, the rate of increase during the 2 day interval necessarily must have been considerably higher than 45.1 per cent. This increment can be calculated to be 54.5 per cent. Thus, this strain of *Rhizopus* can convert glucose actually at a much higher rate than that apparent from the data. A comparison of the rates of acid formation is presented in figure 1.

Alcohol is produced in *Rhizopus nigricans* cultures during the early period of growth and decreases in the very old stages with a coincident increase in fumaric acid (Butkewitsch and Federoff, 1930); this may explain the higher conversion rates. In order to demonstrate that a high rate of conversion could be obtained from alcohol, *Rhizopus* pellicles were allowed to act on ethyl alcohol. As much as 70 per cent of the alcohol consumed was found to have become converted to fumaric acid, thus establishing beyond doubt the ability of the organism to form fumaric acid from ethyl alcohol and the probability that the latter is the intermediary product in the formation of this acid.

The negative value for fumaric acid production obtained in the zinc cultures, during the 7 to 10 day period, may possibly be due to actual consumption of the acid by the fungus, as suggested by Butkewitsch and Federoff (1930) or to its transformation into malic acid (Bernhauer and Thole, 1936). To test this, the

TABLE 5

Effect of trace elements upon growth and acid production by Rhizopus at different periods of growth

Treatment, mgm. per liter	0			10Zn			10Fe			10Fe + 5Mo + 5Mn		
Age of culture, days	5	7	10	5	7	10	5	7	10	5	7	10
Glucose consumed, mgm.	1,368	3,167	4,756	2,375	4,236	4,938	1,394	2,675	4,438	1,308	2,691	4,284
Fumaric acid found, mgm.	463	1,473	1,898	536	719	629	503	1,201	2,054	420	1,258	2,210
Conversion, per cent	33.8	46.5	39.9	22.6	17.0	12.8	36.1	44.9	46.3	32.1	46.8	51.6
Nitrogen consumed, mgm.	18.0	21.5	42.5	28.8	64.4	74.7	17.1	18.9	30.7	13.8	18.6	29.3
Total calcium in solution, mgm.	184.0	506.5	657.0	241.6	397.2	440.0	192.3	408.1	712.9	184.8	421.0	749.6
Calcium due to organic acids, mgm.	158.3	475.7	596.2	200.5	305.1	333.4	167.8	381.1	668.8	165.1	414.4	707.9
Calcium as fumaric acid,* per cent	104.9	107.0	110.0	92.3	81.8	66.3	103.7	108.6	105.4	97.0	105.0	108.0

* The values above 100 per cent are due to incomplete neutralization of the acid by $CaCO_3$; later experiments showed them to be close to 100 per cent.

TABLE 6

*Effect of trace elements during the growth of Rhizopus upon fumaric acid production by pellicles of different ages**

Treatment, mgm. per liter	0			10Zn			10Fe			10Fe + 5Mo + 5Mn		
Age of culture, days	5	7	10	5	7	10	5	7	10	5	7	10
Glucose consumed, mgm.	3,433	7,057	7,783	7,907	12,877	12,291	3,160	5,724	7,626	2,317	5,032	5,924
Fumaric acid found, mgm.	1,676	2,877	2,732	3,646	4,380†	3,988†	1,285	2,643	3,104	997	2,389	2,613
Conversion, per cent	48.8	40.8	35.1	46.1	34.9	32.4	40.7	46.2	40.7	43.3	47.5	44.1
Total calcium in solution, mgm.	522.6	994.5	973.0	1,301.5	1,587.3	1,411.1	505.5	946.6	1,123.3	376.6	873.4	955.3
Calcium as fumaric acid, per cent	92.5	99.7	97.0	97.0	95.3	97.7	87.0	96.4	96.3	91.7	94.4	94.8

* Incubation of pellicles was 5 days in all cases.

† Abundant crystallization of Ca fumarate occurred on the 5th day.

organism was grown on 1 per cent fumaric acid as the only source of energy. One-half of the solution was neutralized with NH_4OH and the other boiled with $CaCO_3$ and filtered. Each portion was divided into 2 lots, only one receiving $ZnSO_4 \cdot 7H_2O$, at the rate of 10 mgm. per liter. Definite growth was obtained in all cases, but it was slow and scant so that it appears that fumaric acid could serve only as a very poor source of energy; no difference was apparent due to zinc. These results suggest the possibility that the reduced yields of fumaric acid in the zinc cultures are not

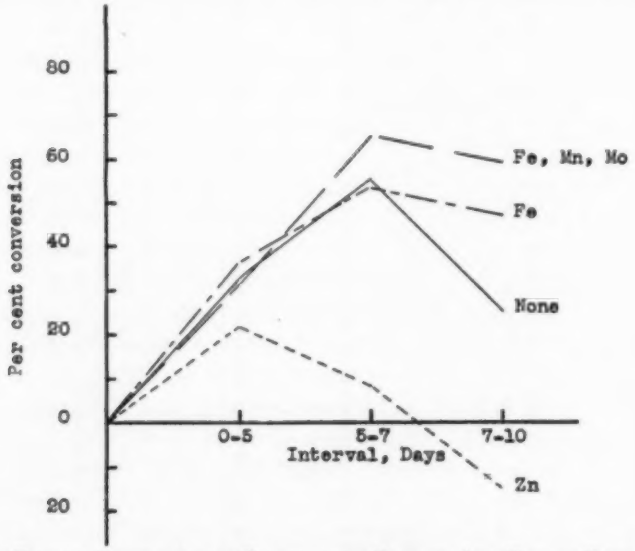

FIG. 1. RATE OF CONVERSION OF GLUCOSE INTO FUMARIC ACID DURING DIFFERENT STAGES OF GROWTH

due to the actual consumption of the preformed fumaric acid but of its precursor and also to its transformation into another acid.

The results on the action of the pellicles from the experiment reported in table 5 are presented in table 6. The zinc pellicles behaved in two respects differently from those of the previous experiment. These pellicles, far heavier than those previously used, consumed glucose at a much higher rate, without, however, any appreciable increase in the quantity of acids other than

fumaric. An explanation of these contrasting results is lacking at present, but it may be associated with the size of the pellicle and glucose concentration. Whereas maximum growth of the organism, as measured by glucose consumption and by nitrogen uptake, was obtained in 10 days, the greatest pellicle activity was found in the case of the 7 day old pellicles. There is, therefore, a period of physiological youth for the fungus which occurred between the 5th and 7th days of growth. This is clearly brought out in figure 1.

Antagonistic actions between iron and zinc on *Aspergillus* was indicated by Roberg (1928, 1931) and Bortels (1927); iron reduced the toxic effect of high and even optimal (Gollmick 1936) concentrations of zinc on sporulation. Since glucose concentration is important in controlling fumaric acid production, it is evident that these three factors govern the relation between growth and fumaric acid formation by *Rhizopus*. An experiment was therefore set up to elucidate these interrelationships. Three series of flasks received the usual basal medium with the following concentrations of glucose: 2.5, 5, 10, 15, 20 and 30 per cent. One series received iron, the second iron and zinc, the third zinc alone as 10 mgm. per liter of salt. All treatments were carried out in duplicate. Seven day cultures were analyzed (table 7).

TABLE 7

Influence of zinc, iron and glucose concentration upon growth and fumaric-acid formation by Rhizopus

GLUCOSE CONCENTRATION	GLUCOSE CONSUMED			FUMARIC ACID PRODUCED			CONVERSION			NITROGEN CONSUMED		
	Fe	Fe+Zn	Zn	Fe	Fe+Zn	Zn	Fe	Fe+Zn	Zn	Fe	Fe+Zn	Zn
per cent	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*per cent*	*per cent*	*per cent*	*mgm.*	*mgm.*	*mgm.*
2.5	3,414	4,138	4,276	1,567	101	None	45.9	2.5	0.0	24.0	82.9	82.8
5.0	5,309	7,793	8,293	2,035	1,810	1,355	38.3	23.2	16.4	36.8	82.9	83.1
10.0	5,075	12,973	12,969	1,620	2,838	2,326	31.9	21.9	17.9	40.4	81.7	81.2
15.0	5,550	9,850	11,100	834	1,016	893	15.0	10.3	8.0	27.8	74.0	67.5
20.0	7,275	11,850	11,050	501	541	816	6.9	4.6	7.4	26.0	65.3	61.0
30.0	8,100	9,838	9,638	190	None	67	2.3	0.0	0.7		42.0	33.5

By increasing the concentration of glucose, there was an increase in glucose consumption only in the iron-containing cultures; this cannot be directly associated with increments in growth, for maximum growth, as measured by nitrogen assimilation, was reached with 10 per cent glucose and then dropped off. Above that concentration, a large part of the glucose was changed to alcohol, reference being previously made to the high fermenting capacity of the iron cultures. Glucose was repressive to acid formation in direct proportion to its concentration. From a yield of 45.9 per cent with the lowest concentration of glucose, the production of fumaric acid was reduced to a mere trace with the highest concentration. The optimum concentration of glucose for the activities of *Rhizopus*, in the presence of an iron salt, therefore depends upon the physiological unit of measurement.

The 10 per cent sugar cultures with zinc alone gave the maximum response in glucose consumption, in growth, in yield of fumaric acid and in per cent conversion. Above and below this concentration, there was a falling off, and, as found previously, no fumaric acid was formed in the 2.5 per cent glucose cultures. The high concentrations of glucose, here as well, blocked almost completely the formation of fumaric acid.

Attention has been called earlier to the fact that there exists a critical carbohydrate-nitrogen ratio, below which fumaric acid is not formed in Zn cultures. This experiment gives evidence of the existence of two carbohydrate-nitrogen ratios; no acid is formed below one and above the other. The $\frac{\text{carbon}}{\text{nitrogen}}$ ratios are calculated to be 25:1 and 300:1, respectively; only within these limits was fumaric acid produced under the experimental conditions.

The combination of iron and zinc was similar to the zinc series, with respect to the maximum activity at the 10 per cent glucose level. The evidence that the antagonistic effects of these two elements partially neutralize one another is quite definite. For example, the favorable influence of iron on fumaric acid formation was clearly manifested with the 2.5 per cent glucose concentration, for the acid was definitely formed under these conditions

and not in the corresponding zinc cultures. Similarly, with the 5, 10, and 15 per cent glucose concentrations, the net result was plainly that of neutralization; the values were increased and reduced as compared with the zinc and iron cultures, respectively.

There is also a distinct associative effect. In the 20 per cent glucose concentration, the presence of both Fe and Zn gave a definite lowering in acid yield and with 30 per cent they completely repressed the formation of the acid while it was still formed in the zinc cultures. The net effect, therefore, of iron and zinc upon fumaric acid production by *Rhizopus* may be either antagonistic or associative, depending upon the sugar concentration; up to 20 per cent glucose, the effect was antagonistic; above this concentration, it was associative.

DISCUSSION

Zinc is indispensable for *Rhizopus* and, on this basis, may be called a "nutrient" according to Steinberg (1934). However, iron and manganese are also essential, but their effect is not comparable to that of zinc under the conditions used. Thus, the term "nutrient" does not serve to indicate the rôle of the particular element in the nutrition of an organism. An element may be vital to the organism in one of two ways—structurally or functionally. Structurally it acts as a building block in the formation of cellular material—it comprises the unit of which cell substance is composed. In this category one usually places carbon, nitrogen, hydrogen, oxygen, sulfur and phosphorus. On the other hand, a substance becomes functional when it is indispensable to some specific process or transformation carried out by the cell substance. Enzymes and catalysts belong here. In some cases, an element may serve in both capacities, as claimed by Buromsky (1936) for potassium and magnesium.

The specific effect that zinc produces on *Rhizopus* is very similar to the functional or catalytic type of nutrient. The greater efficiency of energy utilization by the organism in the presence of this element results in the conversion of a large part of the consumed glucose into cell substance, and only a small portion converted to organic acids. The reduction in the economic coeffi-

cient $\left(\frac{\text{wt. sugar consumed}}{\text{wt. fungus growth}}\right)$ in the presence of zinc is similar to that of *Aspergillus niger* (Buromsky, 1913; Butkewitsch and Orlow, 1922; Ono, 1900). With 2.5 per cent glucose in the medium, *Rhizopus nigricans* consumed all the sugar but produced no fumaric acid, which is similar to the results of Butkewitsch and Orlow (1922) with oxalic acid in *A. niger* cultures.

If zinc were a structural element it would cause merely an increase in the *rate* of utilization of the glucose but no change in the *manner* of utilization of the sugar molecule, i.e., acid production per unit of glucose consumed would be the same. This clearly is not the case here. The formation of considerable amounts of other acidic constituents under the influence of zinc is further indication of the striking modification induced by this element. This specific effect of zinc appears to be sufficient justification for characterizing it as a biological catalyzer, according to the conception of Javillier. If it could be replaced by other heavy metals it would lose its characterization as a true physiological catalyst and would be considered simply as a mere excitant of protoplasmic phenomena. These results, however, are not in agreement with the findings of Bernhauer and Thole (1936), who included zinc in the culture medium and obtained increased yields of fumaric acid by *Rhizopus nigricans*.

Zinc appears to be closely associated with the breakdown of the carbohydrate. This view is supported by the increased efficiency of carbon utilization in the presence of zinc; also by the fact that, in some instances, pellicle cultures, in the absence of nitrogen and minerals and with no apparent increment in growth, gave a reduced proportion of fumaric acid to total acids similar to that of the cultures during the growth period. Further indication of this is shown by the fact that the most marked effect of zinc occurred after the fungus reached maturity when presumably nitrogen metabolism would not be very active while carbohydrate breakdown was still pronounced. Butkewitsch (1922) and Wassiljew (1927, 1930) likewise concluded that zinc is concerned with carbohydrate metabolism.

Wassiljew (1935) further demonstrated that the effect of zinc

was not the same for biochemically different strains of *Aspergillus niger* and he went as far as to suggest a characterization of *A. niger* strains based on their reactivity to zinc.

The results of the studies of carbohydrate concentration indicate that this is an important feature in mold metabolism, especially when quantitative relations between sugar used and end products obtained are under consideration. This is especially true of those fungi which are susceptible to the influence of the heavy metals, but unfortunately this fact has seldom been considered. Unless special purification methods have been employed, it is not unlikely that the combination of these factors could influence the metabolism of the organism so as to give divergent and irreproducible results.

The accumulated evidence suggests that the requirement by filamentous fungi of traces of heavy metals, such as zinc, iron, manganese, copper and possibly others, is a general phenomenon in their nutrition. For the most part this has been proven by observing weight yields and sporulation of the fungi under highly controlled conditions. However, it must be emphasized that only by resorting to physiological studies can the true function of trace elements in the metabolism of fungi be elucidated.

SUMMARY

Zinc was found to be indispensable for the growth of *Rhizopus nigricans*. The presence of 1.2 p.p.m. of zinc ion in an otherwise complete nutrient medium markedly altered the physiology of the fungus. The organism utilized the available energy 3 times more efficiently when zinc was added to the medium. In the absence of added zinc, *Rhizopus* did not completely oxidize the glucose but converted a large part of it into fumaric acid, and made a relatively slow and poor growth. In the presence of zinc, growth was rapid and abundant, a much smaller part of the energy of the glucose being left in the form of fumaric acid. The function of zinc is interpreted as catalyzing a more complete destruction of the glucose molecule with a consequent greater efficiency of energy utilization by the fungus.

The effect of iron in the metabolism of *Rhizopus* was practically the opposite of that of zinc.

Glucose concentration was found to be an important factor governing the influence of zinc and iron. Maximum response to zinc in glucose consumption, growth, absolute yield of fumaric acid and per cent conversion was obtained with 10 per cent glucose medium. In zinc cultures a critical carbon:nitrogen ratio was found to exist. Below a C:N ratio of 25:1 and above 300:1 no fumaric acid was found. Up to 20 per cent glucose concentration, the net effect of iron and zinc was antagonistic; above this concentration, it was associative. Evidence is presented indicating that zinc is closely associated with carbohydrate dissimilation.

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EFFECT OF ALDEHYDES AND FATTY ACIDS AS ADDED HYDROGEN ACCEPTORS ON THE FERMENTATION OF GLUCOSE BY AEROBACTER INDOLOGENES

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Received for publication November 28, 1938

The use of suitable hydrogen acceptors added to a normal fermentation may permit of certain conclusions as to the normal path of fermentation, especially if the acceptor can be traced and the effects of its reduction on the normal dissimilation observed.

Harden and Norris (1902) reported the formation of butylene glycol by *Bacillus lactis-aerogenes* (*Aerobacter aerogenes*) from acetaldehyde in a 1 per cent peptone medium in the absence of glucose. However, most of the acetaldehyde was recovered as acetic and succinic acids and ethyl alcohol.

Neuberg and Kobel (1925) noted that 1 molecule of acetaldehyde added to yeast preparations in the presence of glucose or pyruvic acid condensed with a molecule of acetaldehyde biologically formed to yield acetylmethylcarbinol.

Kluyver and Donker (1925) were able to cause yeast and lactic acid bacteria to form acetylmethylcarbinol from glucose when methylene blue or sulphur was added as a hydrogen acceptor.

Whetham (1927) believed the presence of acetylmethylcarbinol was a reliable indication of the intermediary occurrence of acetaldehyde.

Hammer (1936) found that the addition of homologs of acetaldehyde to fermenting milk cultures of *Streptococcus liquefaciens* resulted in an increase in acetylmethylcarbinol but that homologs of acetylmethylcarbinol were not formed.

Reynolds, Jacobsson and Werkman (1937) noted that acetic

acid, when added to a glucose fermentation by *Aerobacter indologenes*, was reduced and the yield of hydrogen suppressed. The reduced acetate was quantitatively accounted for as 2,3-butylene glycol.

The purpose of the present study was to determine the effect of homologs of acetaldehyde and acetic acid on the fermentation of glucose by *Aerobacter*. The results should be of considerable importance to an understanding of the formation of acetylmethylcarbinol and 2,3-butylene glycol. If a homolog of acetic acid, e.g., propionic, were reduced and the product condensed to the carbinol and further reduced to glycol, it is reasonable to expect that 4-carbon-, 5-carbon- and possibly 6-carbon-glycols would be formed. Since, on oxidation the glycols yield the corresponding acids from which they are derived, it is possible to determine whether such a condensation occurs. Likewise for higher aldehydes a similar procedure can be applied. Use of the higher acids and aldehydes offers an advantage over acetic acid and acetaldehyde in that their reduction products can be traced.

In the results to be reported, acetaldehyde, propionaldehyde, butyraldehyde and acetic, propionic, butyric and fumaric acids were added to glucose fermentations and their effect on dissimilation studied.

METHODS

Fermentations were conducted at 30°C. in 500 ml. Erlenmeyer flasks fitted with dropping funnels for the addition of the acids and aldehydes. The medium was 2.0 per cent glucose, 0.3 per cent $(NH_4)_2SO_4$, 1.0 per cent K_2HPO_4 and 0.05 per cent peptone. Ten per cent (by volume) of tap water was added for the salts present. The medium was autoclaved and after inoculation flushed with oxygen-free nitrogen before connecting to the bottles for the absorption of gases.

Freshly distilled aldehydes were added in approximately 0.6 M concentration. The added aldehyde was determined by titration of bound bisulphite with iodine according to Donnally (1933).

Acids used were weighed, neutralized to bromothymol blue, made up to the desired concentration and determined by steam-distilling an aliquot part and titrating.

The fumaric acid was weighed, neutralized, sterilized by Seitz filtration and determined gravimetrically as the silver salt.

The inoculum was prepared by growing *Aerobacter indologenes* on nutrient agar and using an aqueous suspension for inoculation. Cultures were examined before and after fermentation by the gram stain.

Carbon dioxide and hydrogen were collected in bottles containing 1 M alkali, the carbon dioxide being absorbed in the alkali and the hydrogen determined by volume displacement. Residual carbon dioxide was determined gravimetrically by acidifying the medium to congo red and refluxing an aliquot part in a stream of CO_2-free air.

After removal of carbon dioxide the sample was distilled to half volume, the distillate neutralized and again distilled to half volume to recover alcohols. The two residues were combined, acidified to congo-red paper and steam-distilled to collect volatile acids. The volatile acids in the distillate were determined by the partition method according to Osburn, Wood and Werkman (1933). Formic acid was determined by the method of Auerback and Zeglin (1922).

Alcohols were determined by oxidizing an aliquot part of the neutral volatile distillate with H_2SO_4—$K_2Cr_2O_7$ mixture in a closed flask in a boiling water bath, steam-distilling and determining the resulting acids on the steam distillate by the partition method.

Acetylmethylcarbinol was determined according to Stahly and Werkman (1935). 2,3-Butylene glycol was separated from the medium by steam distillation from a neutral medium saturated with $MgSO_4 \cdot 7H_2O$. The glycol was determined on the distillate by oxidizing with periodic acid and directly distilling the resulting acetaldehyde into sodium bisulphite solution and titrating with iodine.

Lactic acid was determined on the residue of the butylene

glycol distillation by the method of Friedemann and Graesser (1933).

Residual aldehydes were determined by distilling an aliquot part of the fermentation directly into bisulphite and titrating with iodine.

To determine whether any of the added aldehydes or acids had condensed to the corresponding glycols, the glycol distillate was oxidized, as in the procedure for determining alcohol, and the resulting steam distillate partitioned to detect the presence of acids other than acetic. By this procedure butylene glycol is oxidized quantitatively to acetic acid, each mole yielding two of the acid.

RESULTS

It was first desirable to determine the fate of acetaldehyde added to fermenting cultures of *Aerobacter indologenes* under acid and alkaline conditions (table 1). It has previously been shown (Mickelson and Werkman, 1938; also see table 1) that the pH of the medium has a very pronounced effect on the formation of butylene glycol. The addition of acetaldehyde to an alkaline fermentation results in its equimolar conversion into acetic acid and ethyl alcohol. The work of Reynolds and Werkman (1936) suggested that acetic acid may be an intermediate in the formation of 2,3-butylene glycol with acetaldehyde and acetylmethylcarbinol acting as intermediates in the transformation. If *Aerobacter indologenes* contains carboligase which will couple acetaldehyde under conditions when added acetic acid is not attacked (alkaline reaction), it should be possible to demonstrate whether acetic acid is necessary in the formation of butylene glycol. The results obtained do not bear out this hypothesis (table 1).

When the aldehyde is added to a fermentation conducted at a pH level favorable to the formation of butylene glycol there is a substantial increase in acetylmethylcarbinol (table 1) and likewise ethyl alcohol and acetic acid. It appears that the added aldehyde has been in part condensed to the carbinol. Since acetaldehyde can serve as a precursor of acetic acid,

TABLE 1

Effect of adding aldehydes to glucose fermentations of Aerobacter indologenes

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Effect of adding aldehydes to glucose fermentations of Aerobacter indologenes

	ALDEHYDE FERMENTED	CO_2	H_2	FORMIC ACID	ACETIC ACID	LACTIC ACID	ACETYLMETHYL-CARBINOL	2,3-BUTYLENE GLYCOL	ETHYL ALCOHOL	PROPYL ALCOHOL / BUTYL ALCOHOL	SUCCINIC ACID	BUTYRIC ACID	CARBON RECOVERY	O/R INDEX
													per cent	
Control	0	164.00	41.50	16.40	0.66	4.30	1.15	64.50	58.20				95.5	0.98
Acid acetaldehyde	43.20	174.00	8.46	0.76	9.13	2.64	17.66	66.40	63.80				96.7	1.06
Alkaline acetaldehyde	43.00	10.10	2.73	167.20	109.80	8.80	0	1.36	109.30				94.3	1.03
Propionaldehyde	24.20	173.50*	41.20*	8.65	1.00	3.70	0.32	68.00	32.40	27.20 (PROPYL ALCOHOL)	5.15		93.5	1.05
Butyraldehyde	22.50	175.00	41.20	13.00		4.32	0.65	65.60	50.40	14.40 (BUTYL ALCOHOL)	3.52	5.65	97.5	1.01

Products in mM per 100 mM fermented glucose.

* Calculated, sample lost.

TABLE 2

Effect of adding acids to glucose fermentations of Aerobacter indologenes

	ACID FERMENTED	CO_2	H_2	FORMIC ACID	ACETIC ACID	LACTIC ACID	ACETYLMETHYL-CARBINOL	2,3-BUTYLENE GLYCOL	ETHYL ALCOHOL	PROPYL ALCOHOL	SUCCINIC ACID	ACETIC ACID ADDED	CARBON RECOVERY	O/R INDEX
Control	0	164.00	41.50	16.40	0.66	4.30	1.15	64.50	58.20				95.5	0.98
Acetic acid	36.00	174.00	0	9.00	50.00	3.00	2.20	88.00	55.00			86.00	98.6	0.95
Propionic acid	27.50	163.00	14.90	17.70	8.60	2.70	0.64	75.00	21.60	27.50			93.0	1.02
Fumaric acid	18.50	165.20	35.60	17.50	0.67	2.92	0.32	66.00	48.50		24.10		97.0	1.01

Products in mM per 100 mM fermented glucose.

ethyl alcohol, acetylmethylcarbinol or butylene glycol, there is no assurance that condensation has occurred because the added aldehyde has not been tagged and its fate determined.

Higher aldehydes and acids offer the advantage that it is possible to trace them. If synthetic benzaldehyde is coupled with biologically formed acetaldehyde to give phenylacetylcarbinol as Neuberg and Ohle (1922) found to be the case with yeast, it may be expected that *Aerobacter* will couple added propionaldehyde or butyraldehyde with acetaldehyde to give the corresponding carbinols. The data show that such is not the case. Instead, the added aldehydes are oxidized and reduced to the corresponding acids and alcohols with no increase in acetylmethylcarbinol. These results are to be compared with those of Hammer (1935) who obtained increases in acetylmethylcarbinol by the addition of homologs of acetaldehyde to cultures of *Streptococcus liquefaciens*. Likewise, those substances which do not cause increased yields of the carbinol or glycol with *Aerobacter* do not decrease the yield of hydrogen as do acetaldehyde and propionic and acetic acids.

Most of the work on the origin of acetylmethylcarbinol in bacterial and yeast fermentations has emphasized that one mole of the acetaldehyde must be nascent, i.e., of biological origin. Tomiyasu (1936) claims to have obtained a synthesis of acetylmethylcarbinol with a yeast preparation from synthetic acetaldehyde and states that nascent aldehyde from sugar or pyruvic acid is unnecessary. Recent work by Discherl (1937) points to the necessity of having nascent aldehyde formed before acetylmethylcarbinol synthesis can take place from synthetic acetaldehyde. He attributes the results of Tomiyasu to the lack of a yeast preparation free of endogenous fermentation.

When acetaldehyde was added to cell suspensions of the organism in buffer at a pH favorable to the formation of acetylmethylcarbinol and 2,3-butylene glycol from glucose no condensation of aldehyde occurred. Apparently the formation of acetylmethylcarbinol, if it occurs by a condensation of acetaldehyde, is an energy-consuming reaction and, since the side reaction providing the energy is not taking place, no formation of

acetylmethylcarbinol is possible. It is also possible that the acetaldehyde must couple with some other intermediate in order that the formation of the carbinol may occur.

If the cells cannot synthesize acetylmethylcarbinol from synthetic acetaldehyde it is not readily conceivable that a similar compound of biological origin would be more readily converted. Perhaps, as suggested by Discherl (1931), the acetaldehyde couples first with pyruvic acid and yields acetylmethylcarbinol with a loss of carbon dioxide. At any rate it may not be necessary that the compound of biological origin be nascent acetaldehyde.

Propionaldehyde and butyraldehyde do not cause increases in acetylmethylcarbinol or butylene glycol. In either case there is a small amount of succinic acid formed, accompanied by a decrease in ethyl alcohol (table 1). Propionaldehyde caused a greater reduction in the yield of ethyl alcohol and was itself entirely converted to propyl alcohol. With butyraldehyde some butyric acid was formed.

Unless *Aerobacter indologenes* is unable to synthesize higher glycols, it is not unreasonable to suppose that acetaldehyde is reduced to the alcohol and does not directly enter into the formation of butylene glycol. This is in agreement with the results obtained by Hammer in the case of most of the aldehydes tried with *S. liquefaciens*.

Propionic acid apparently produces the same effect as acetic acid, i.e., causes an increase in butylene glycol, a reduction in the yield of gaseous hydrogen and is itself reduced. It is, however, not attacked quite as vigorously as acetic acid. Propionic acid, though reduced, does not itself directly enter into the formation of butylene glycol. By being reduced to propyl alcohol it causes a reduction in ethyl alcohol and diverts some of the two-carbon compound which ordinarily would go to ethyl alcohol to butylene glycol or acetic acid. These results have been confirmed by repeated experiments. These data indicate that acetic acid may act similarly when added, i.e., be reduced to ethyl alcohol, cause a decrease in gaseous hydrogen and an increase in butylene glycol without itself being transformed to the glycol. When conditions are optimum for the reduction

of acetic and the formation of butylene glycol, acetic acid may not be formed.

Fumaric acid was used to determine whether the results were merely the effect of an added hydrogen acceptor. According to Barker (1936), for each mole of fumaric acid oxidized by *Aerobacter aerogenes* another acts as a hydrogen acceptor to yield succinic acid. Fumaric acid has no effect on the formation of butylene glycol. It causes a slight decrease in hydrogen and is itself reduced to succinic acid. However, more succinic acid is formed than can be accounted for by the fumaric added; the decrease in ethyl alcohol accounts for the added increase in succinic. Succinic acid was identified as the p-nitrobenzyl ester.

In several experiments butyric acid has not been found to act as a hydrogen acceptor.

SUMMARY AND CONCLUSIONS

Acetic and propionic acids, when added to glucose fermentations by *Aerobacter indologenes*, are reduced, resulting in an increase in butylene glycol and a decrease in gaseous hydrogen. Propionic acid is reduced to propyl alcohol.

Acetaldehyde undergoes a Cannizzaro reaction when added to an alkaline glucose fermentation by *Aerobacter indologenes*. When added to a fermentation with a reaction favorable to the formation of butylene glycol, increases are noted in acetylmethylcarbinol and ethyl alcohol and decreases in hydrogen.

Propionaldehyde and butyraldehyde are reduced to the corresponding alcohols with some of the butyraldehyde going to butyric acid. No decrease in hydrogen is observed but slight increases in carbon dioxide and the formation of small amounts of succinic acid with decreases in ethyl alcohol are noted.

Fumaric acid is reduced to succinic acid and some of the glucose carbon is also diverted to form succinic at the expense of the ethyl alcohol.

Acetaldehyde is not coupled to form acetylmethylcarbinol by *Aerobacter* at a pH high enough to prevent formation of the latter from glucose.

Since propionic acid produces an effect similar to acetic acid

and does not result in the formation of a higher homologous glycol it is doubtful whether acetic acid is a necessary intermediate in the formation of butylene glycol.

It is suggested that perhaps synthetic acetaldehyde, when added, is coupled with some other intermediate than acetaldehyde during the formation of acetylmethylcarbinol.

The mechanism of formation of acetylmethylcarbinol by *Aerobacter* is still an open question.

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THE PREPARATION OF PERSEULOSE BY OXIDATION OF PERSEITOL WITH ACETOBACTER SUBOXYDANS[1]

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Contribution from the National Institute of Health, U. S. Public Health Service

Received for publication December 7, 1938

The present work was undertaken at the suggestion of Dr. C. S. Hudson and Dr. Raymond M. Hann for the purpose of improving the method for preparing the rare ketoheptose perseulose, which was needed for chemical studies.[2] The best starting material is unquestionably perseitol, which occurs in the avocado (*Persea gratissima*); a large supply of this crystalline polyhydroxy alcohol was kindly furnished by Dr. A. E. Knauf of this Institute, who had isolated it as a by-product in the preparation of D-mannoheptulose from avocados. Perseulose was discovered by Bertrand (1908) through the oxidation of perseitol by *Acetobacter xylinum*. The experience of Wells, Stubbs, Lockwood and Roe (1937) has indicated that *Acetobacter suboxydans* in submerged growth gives a nearly quantitative conversion of sorbitol to sorbose, and with the coöperation and technical assistance of the above mentioned colleagues of the Industrial Farm Products Research Division of the Bureau of Chemistry and Soils, U. S. Department of Agriculture, conditions have been determined for the quantitative biochemical oxidation of perseitol to perseulose.

Bertrand used 0.5 per cent yeast extract as the only nutrient substance in the culture medium, other than perseitol, the latter being employed in 2 per cent concentration. The oxidation in

[1] Publication authorized by the Surgeon General, U. S. Public Health Service.

[2] The following publications have been made: Hann, R. M., Tilden, E. B., and Hudson, C. S., J. Am. Chem. Soc., 1938, **60**, 1201. Hann, R. M., and Hudson, C. S., Ibid., 1939, **61**, 336. Richtmyer, N. K., Hann, R. M., and Hudson, C. S., Ibid., 1939, **61**, 340.

these surface cultures required several weeks, and the yield of the sugar was about 45 per cent of the theoretical. Bertrand recovered the sugar by concentrating the culture fluid, after lead treatment, and extracting the syrup with boiling 90 per cent alcohol, which dissolved the sugar and precipitated the unoxidized perseitol. The sugar crystallized in a mass from the concentrated alcoholic solution. After two or more recrystallizations from alcohol, the sugar had an optical rotation at equilibrium of $-81°$.

Bertrand's (1898) sorbose bacterium, which he had obtained from the skin of the berries of the mountain ash (*Sorbus aucuparia*), was later found to be identical with *Acetobacter xylinum*, which had been isolated from vinegar by Adrian Brown (1886). Kluyver and DeLeeuw (1924) found in beer a new member of the group of oxidizing bacteria, which they named *Acetobacter suboxydans*. This organism was found by Fulmer, Dunning, Guymon, and Underkofler (1936) to give better results than *A. xylinum* in the preparation of sorbose, which has become of great importance as the starting point for the synthesis of ascorbic acid, and Wells, Stubbs, Lockwood and Roe (1937), as has been mentioned, used submerged growths of *A. suboxydans* in their studies of the optimum conditions for large scale production of sorbose.

In our preliminary attempts to oxidize perseitol, we were more successful with *A. suboxydans* than *A. xylinum*, and the later work was all done with *A. suboxydans*.[3] We have not made a systematic comparison of the perseitol-oxidizing ability of the two organisms under the most favorable conditions.

EXPERIMENTAL DATA

Perseitol is not very soluble at room temperature, and not more than 3 to 4 per cent could be used without its crystallizing from solution during the early part of the incubation. We used

[3] The culture of *A. suboxydans* was kindly furnished by Dr. L. B. Lockwood of the Division of Industrial Farm Products Research. The *A. xylinum* culture was obtained from the American Type Culture Collection.

3 per cent in most of the experiments. Difco yeast extract, 0.5 per cent, constituted the basic medium. Preliminary experiments with surface cultures were made in 125 ml. Erlenmeyer flasks containing 20 ml. of the medium. *A. suboxydans* was maintained on agar slants containing 0.5 per cent yeast extract and 5 per cent sorbitol, and the flasks were inoculated with 1 ml. of aqueous suspensions of such cultures. Two-milliliter samples of medium were withdrawn at intervals and tested for reducing value by the Shaffer-Hartmann method (1921). Bertrand's (1906) figure (100 mgm. perseulose equivalent to 157.3 mgm. copper) was used for approximate calculation until the pure sugar was obtained for construction of a curve of copper values.

In the simple yeast extract medium, which is adequate for sorbitol oxidation, only 30 to 35 per cent of the perseitol was oxidized. The phosphate content of the medium was increased by adding 0.3 per cent acid potassium phosphate (Underkofler and Fulmer, 1937), leading to considerably increased oxidation. No further increase was obtained by aeration of the submerged culture in a Jena glass gas-washing bottle until the inoculum was varied by using 1 ml. of a 48-hour aerated sorbitol culture, when nearly complete oxidation was obtained. That this result was due to some constituent of the sorbitol culture was evident from the fact that subcultures inoculated from the actively oxidizing perseitol culture showed only partial oxidation. We knew that sorbose was present in the sorbitol culture and also probably some glucose, which is contained in crude sorbitol, and on adding small quantities of either of these to the medium the conditions became so favorable that the perseitol was completely oxidized. These preliminary results are summarized in table 1. Five-hundredths per cent glucose appeared to be slightly more favorable than 0.1 per cent sorbose and was thereafter employed regularly in the medium. Subsequent experiments (Hann, Tilden and Hudson, 1938) showed that the medium containing glucose and phosphate is a favorable one for the oxidation of a variety of sugar alcohols other than perseitol.

TABLE 1

Determination of conditions for oxidation of perseitol

FLASK NUMBER	MEDIUM CONTAINING 3 PER CENT PERSEITOL	VOLUME	INOCULUM	AERATION	TEMPERATURE OF INCUBATION	TIME OF INCUBATION	PER CENT SUGAR IN MEDIUM* AFTER INCUBATION
		ml.			*degrees*	*days*	
1	0.5 per cent Difco yeast extract	20	1 ml. aqueous suspension (20 ml. water from 2 sorbitol agar slants)	None	20	5	0.9
					30	5	1.0
					20	9	1.2
					30	9	1.38
2	Same +0.3 per cent KH_2PO_4	20	Same	None	20	5	1.35
					30	5	1.4
					20	9	1.8
					30	9	1.9
3	Same as 2, +0.0003 M $MgSO_4$	20	Same	None	20	5	1.39
					30	5	1.35
					20	9	1.8
					30	9	2.0
4	Same as 3, +0.05 per cent glucose	20	Same	None	20	5	1.37
					30	5	1.41
					20	9	2.8
					30	9	2.8
BOTTLE NUMBER							
1	0.5 per cent Difco yeast extract +0.3 per cent KH_2PO_4	200	1 ml. from 48-hour aerated fluid sorbitol culture	200 ml. per min. under 8 pounds pressure	30	7	3.0
2	Same	200	1 ml. from 48-hour aerated fluid perseitol culture	Same	Same	Same	1.2
3	Same +0.1 per cent sorbose	200	Same	Same	Same	Same	2.6
4	Same as 2, +0.05 per cent glucose	200	Same	Same	Same	Same	3.3
5	Same as 3	200	1 ml. aqueous suspension, as for flasks 1–4	Same	Same	Same	3.1
6	Same as 4	200		Same	Same	Same	3.4

* Calculated for reducing values by Shaffer-Hartmann test, using Bertrand's figures for copper equivalents. For preincubation values see tables 3 and 4.

Recovery of the sugar

One liter of pooled bottle cultures containing reducing sugar equivalent to 3 per cent was clarified by filtration through decolorizing carbon (Darco G60) and the clear filtrate concentrated *in vacuo* at 35 to 40° to a thick syrup, which crystallized spontaneously after 48 hours. The separated crystals, about 15 grams, were recrystallized from 15 parts of 95 per cent alcohol, and yielded 12 grams of perseulose hemihydrate of melting point 106° and $[\alpha]_D^{20}$ −81.3° in water at equilibrium. This material was used for the determination of copper equivalents by the Shaffer-Hartmann technique (table 2). An additional 7.5 grams was subsequently obtained from the syrup, making the total yield about 65 per cent. An alternative method of crystallizing

TABLE 2

Reduction values of perseulose hemihydrate by the Shaffer-Hartmann method

100 mgm. perseulose hemihydrate reduced	166.4 mgm. copper
70 mgm. perseulose hemihydrate reduced	118.0 mgm. copper
50 mgm. perseulose hemihydrate reduced	83.9 mgm. copper
20 mgm. perseulose hemihydrate reduced	31.2 mgm. copper

the ketose, which gives a higher yield, is described by Hann and Hudson (1939).

Oxidation in rotary drum fermenters

We were fortunate in having put at our disposal on three occasions one or more of the rotary drum fermenters designed by Herrick, Hellbach, and May (1935) and used in the preparation of sorbose and of gluconic acid (Wells, Moyer, Stubbs, Herrick, and May, 1937). Except for the additions to the culture medium already indicated, the conditions employed for sorbitol oxidation were found to be satisfactory for perseitol, though the reaction time was somewhat longer. At the suggestion of Wells and his collaborators,[4] neutralized corn-steep liquor, 0.3 per cent, replaced the yeast extract in these experiments, having been proved satisfactory by bottle culture tests. Three per cent perseitol was used in the first two drums, 4 per cent

[4] Ind. Eng. Chem. In press.

incubation values see tables 3 and 4.

in drums 3 to 6. The volume of culture in each drum was 3200 ml. except in drum 1, in which it was 2000 ml. The inoculum was varied somewhat in the latter four drums, which were run at the same time. Oxidation was complete in all, but much more rapid with a large inoculation (drum 6) than with a relatively small one (drum 5), and more rapid when the inoculum was a yeast-extract culture (drum 4) than one grown on steep medium (drum 3). The bacterial count reached 100 million

TABLE 3

Oxidation of 3 per cent perseitol in rotary drum fermenters

DRUM NUMBER	MEDIUM CONTAINING 3 PER CENT PERSEITOL	VOLUME	INOCULUM	AERATION AND AGITATION	TEMPERATURE OF INCUBATION	TIME OF INCUBATION	pH	PER CENT REDUCING SUBSTANCE CALC. AS PERSEULOSE†	
								Before incubation	After incubation
		ml.			*degrees*	*hours*			
1	0.3 per cent purified corn steep liquor*, 0.3 per cent KH_2PO_4, 0.05 per cent glucose	2,000	Bacteria washed from Petroff flasks (5) and agar slants (6) of 5 per cent sorbitol agar	1200 ml. per min. at 30 pounds pressure 13 R.P.M.	30	0	5.3	0.111	
						16	4.6		0.72
						24	5.2		1.79
						36	5.2		3.10
2	Same	3,200	200 ml. 48-hour aerated fluid culture on steep medium containing 3 per cent perseitol	Same	Same	0	5.6	0.34‡	
						21	5.4		1.70
						27	4.6		2.25
						45	4.9		3.05

* Kindly furnished by the Farm Products Research Laboratory.

† Calculated from curve of copper values constructed from reducing values obtained with known amounts of perseulose (see table 2).

‡ Inoculum contained nearly 6 grams of perseulose.

per ml. in all the drums within 5 hours and remained at that figure throughout; dilutions beyond that point were not made. The pH showed a tendency to fall during oxidation, rising to the initial value at completion of the reaction. The data are summarized in tables 3 and 4 and in the chart. The yield of perseulose in these experiments was found to be quantitative (Hann and Hudson, 1938).

The time necessary for the oxidation in the drums (47 hours), as compared with that of sorbitol (16 hours), suggested that the

TABLE 4

Oxidation of 4 per cent perseitol in rotary drum fermenters

DRUM NUMBER	MEDIUM CONTAINING 4 PER CENT PERSEITOL	VOLUME	INOCULUM	AERATION AND AGITATION	TEMPERATURE OF INCUBATION	TIME OF INCUBATION	pH	PER CENT REDUCING SUBSTANCE CALC. AS PERSEULOSE†	
								Before incubation	After incubation
		ml.			*°C.*	*hours*			
3	0.3 per cent neutralized corn steep liquor*, 0.3 per cent KH_2PO_4, 0.05 per cent glucose	3,200	200 ml. 48-hour aerated culture on steep medium containing 3 per cent perseitol	1200 ml. per minute at 30 pounds pressure 13 R.P.M.	30	0	4.9	0.34‡	
						20	4.4		1.05
						27	4.4		1.80
						42	4.9		3.00
						49	5.0		3.40
						66	5.0		3.90
						72	5.1		3.95
4	Same	Same	200 ml. 48-hour aerated culture on yeast extract medium containing 3 per cent perseitol	Same	Same	0	5.1	0.30‡	
						20	4.9		1.65
						27	4.9		3.15
						42	5.2		4.00
						47	5.1		4.15
5	Same	Same	Bacteria from 2 Kolle flasks solid medium (sorbitol agar) washed into 200 ml. steep medium	Same	Same	0	5.1	0.117	
						20	4.6		1.00
						27	4.6		1.90
						42	5.1		3.10
						49	5.1		3.45
						66	5.2		3.85
						72	5.3		3.95
6	Same	Same	Bacteria from 6 flasks solid med. in 200 ml. steep medium	Same	Same	0	5.0	0.133	
						20	4.9		1.90
						27	4.7		2.95
						42	5.1		3.75
						47	5.0		3.95

* Kindly furnished by the Farm Products Research Laboratory.

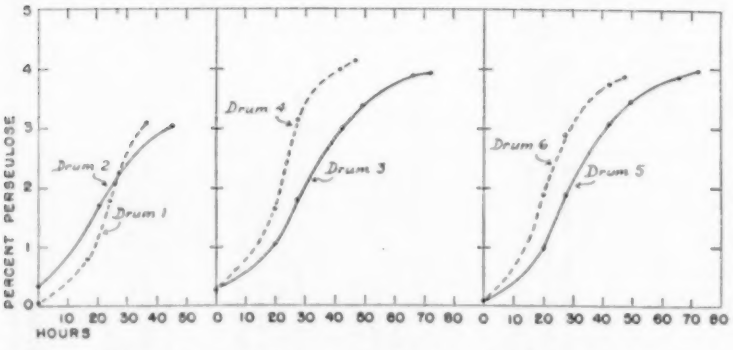
† Calculated from curve of copper values constructed from reducing values obtained with known amounts of perseulose (see table 2).

‡ Inoculum contained nearly 6 grams of perseulose.

conditions might not be ideal, and it was later found that the pH of 5.5 was not the optimum. Surface cultures containing 3 per cent perseitol were set up in 200 ml. lots in 500 ml. Erlenmeyer flasks, in which the potassium phosphate was replaced by various mixtures of dibasic sodium phosphate and monobasic potassium phosphate to give hydrogen ion concentrations ranging from pH 5.9 to 7.3. The salts were made up in M/5 concentration, and 10 ml. of a given mixture were added to each 100 cc. of medium, to give approximately 0.3 per cent phosphate concentration. The pH of the cultures was determined by measure-

CHART 1

OXIDATION OF PERSEITOL IN ROTARY FERMENTERS



ments with the glass electrode. As the figures below show, the optimum pH is 6.4.

Initial pH	*Per cent perseulose calculated from reducing value after 9 and 12 days*
7.3	2.48
6.8	2.89
6.4	2.95
5.9	2.86
5.5	2.54

SUMMARY

The cultural conditions favorable to oxidation of perseitol to perseulose by *Acetobacter suboxydans* are described. A small amount of glucose in the medium (0.05 per cent) was found to promote complete oxidation. Three to four per cent perseitol

is changed quantitatively to perseulose in 36 to 48 hours when the cultures are aerated and agitated. The medium described appears to be favorable for the oxidation of a number of other sugar alcohols.

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THE EVALUATION OF A GROUP OF GERMICIDES BY THE TISSUE CULTURE TECHNIQUE

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Received for publication December 12, 1938

The phenol coefficient test is universally employed for determining the germicidal efficiency of preparations or compounds intended for the destruction of bacteria. The test was originally designed to be used for comparing the toxicity of phenol with phenol-like compounds. In recent years the method has been used to test compounds which are totally unlike phenol, with the result that such evaluations have been the cause of considerable confusion.

As Reddish (1937) stated: "The phenol coefficient test has been widely used for comparing the germicidal efficiency of pure chemical compounds. During recent years, however, there have been a number of instances of departures from the standard phenol coefficient test which have caused considerable confusion. Germicides have been tested by this method which are not chemically related to phenol and whose chemical and germicidal activity differ so greatly from that of this standard germicide that they should not be compared in this way, for example, chlorine compounds, mercury compounds, formalin, hydrogen peroxide, iodine, picric acid, certain essential oils, etc., and a large number of organic compounds."

"Another departure has been the liberties which certain investigators have taken with the test itself, changing it to suit the compounds tested. Many germicides which are soluble in alcohol but not in water, or only slightly so, have been tested by this method in dilutions of alcohol instead of water, although water is specified as the diluting agent in all the standard phenol coefficient tests. Certain other germicides which are soluble in alkali

but not in water, or only slightly so, have been tested in dilutions made in alkali solutions instead of water, and the figures obtained have been designated as phenol coefficients. As a result, so-called phenol coefficients have been reported in the chemical literature which do not represent a true comparison of the germicidal activity of such compounds with phenol, which is water-soluble. The test itself is often blamed when discordant results are reported, whereas the unwarranted modifications made by certain investigators are entirely responsible."

It is generally agreed that a phenol coefficient is of no value in determining the efficiency of a germicide intended for clinical application. A phenol coefficient attempts to compare the toxicity of a germicide on a given organism with that of phenol but gives no information as to its effect on living tissue. It can be seen that a germicide with a high phenol coefficient and a proportionately high toxicity to tissue would have no advantage over one with a low phenol coefficient and a proportionately low toxicity towards living tissue.

In 1935 a new method was proposed (Salle and Lazarus, 1935 a) for the evaluation of germicidal substances intended for clinical use. The germicides were tested for their effect on the growth of living embryonic chick heart tissue as well as for their ability to kill bacteria. A number known as the toxicity index was determined which was defined as the ratio of the highest dilution of germicide required to prevent the growth of the tissue in forty-eight hours to the highest dilution required to kill the test organism in ten minutes. The smaller the toxicity index, the more nearly perfect the chemotherapeutic agent. Theoretically, an index less than one means that the germicide is more toxic to bacteria than to the embryonic tissue while an index greater than one means that the germicide is more toxic to the embryonic tissue than to the bacteria.

The compounds which were rated by the method included Merthiolate (Salle and Lazarus, 1935 a), Metaphen (1935 b), Mercurochrome (1935 c), Hexylresorcinol (1935 d), iodine (1935 e), iodine trichloride (1935 f), potassium mercuric iodide (1935 g), and mercuric chloride (1935 h). The toxicity indices

were later grouped together in one communication (Salle, McOmie and Shechmeister, 1937).

Recently (Salle, McOmie, Shechmeister and Foord, 1938) the method was improved by (*a*) performing all tests at 37°C. (temperature of the human body) instead of at 25°C., (*b*) keeping the period of action of germicide on tissue and bacteria the same, viz., 10 minutes, (*c*) performing all tests in the presence of a definite amount of organic matter. Most of the above named compounds were retested by the improved method and others added (Foord, McOmie and Salle, 1938; Shechmeister and Salle, 1938).

The method is briefly as follows:

EFFECT OF GERMICIDE ON TISSUE

Chick hearts are removed from 10–12 day old embryos and minced into fragments about 0.5 mm. in diameter. The fragments are suspended in Tyrode's solution, about 100 pieces per cubic centimeter. One-half ml. of suspension is pipetted into each of a series of tubes containing 2.5 ml. of a mixture of three parts embryonic extract[1] and one part defibrinated horse serum. The tubes are placed in a 37°C. waterbath.

A series of aqueous dilutions of the germicide is prepared and heated to 37°C. Two milliliters of each dilution are transferred to the tubes containing the tissue-serum-extract mixture.[2] Final volume, 5 ml.

After ten minutes, the tubes are removed from the bath and the germicidal dilutions aspirated from the sedimented fragments. The fragments are resuspended in Tyrode's solution and the supernatant fluid again aspirated. Finally 1 ml. of Tyrode's

[1] The extract is prepared by mincing 12 day old embryos, diluting to five times its volume with Tyrode's solution and centrifugating. The clear supernatant liquid is the extract.

[2] The germicide must be added to the mixture last since this is the order of action in clinical practice. Most germicides are considerably inactivated in the presence of organic matter. If the germicide is added to the fragments first, and then the organic material, no inactivation of the germicide will occur with the result that a smaller concentration of the chemical will produce the same effect.

solution is added to each tube and the fragments embedded in plasma in Carrel flasks. The killing dilution is determined by the absence of proliferating cells after seventy-two hours or longer.

A series of aqueous dilutions of germicide in tubes is also brought to 37°C. Two milliliters of each dilution of germicide are transferred to the tubes containing the bacteria-serum-extract mixture. Final volume, 5 ml.

After ten minutes, the tubes are removed from the bath and a 4 mm. loopful transferred from each tube to broth. To eliminate any bacteriostatic effect, subcultures are made to either 100 ml. amounts of broth or to a 15 ml. tube of broth followed by transfer of 4 loopfuls from this tube to a second one. The killing dilution is determined by the absence of growth in broth subcultures after seventy-two hours or longer.

The phenomenon of bacteriostasis has been the cause of a considerable number of discrepancies in the evaluation of germicides. Iodine, Azochloramid, Hexylresorcinol, phenol and the phenolic compounds were found to be free from any appreciable bacteriostatic action while the mercury and the silver compounds exhibited a considerable effect (Salle, McOmie, Shechmeister and Foord, 1938).

All germicides were tested in aqueous solutions. This eliminated any germicidal effect of alcohol or other toxic solvent.

The killing concentrations of various germicidal preparations for bacteria and tissue and their toxicity indices are given in table 1.

The *Staphylococcus aureus* and *Eberthella typhosa* phenol coefficients have also been included in the table. It may be seen that there is no relationship between the phenol coefficients and their corresponding toxicity indices.

The order in which the germicides fall differs with the two organisms but agrees very closely with the ratings assigned to them in the original method (Salle, McOmie and Shechmeister, 1937).

The results show that the halogens iodine and Azochloramid exhibit the highest degree of germicidal efficiency of the compounds tested by the above technique. They combine low tissue

TABLE 1

Killing concentrations for tissue and bacteria and corresponding toxicity indices and phenol coefficients in 10 minutes at 37°C

COMPOUND	TISSUE (A)	KILLING DILUTION		TOXICITY INDEX = A/B		PHENOL COEFFICIENT	
		S. aureus (B)	*E. typhosa* (B)	*S. aureus*	*E. typhosa*	*S. aureus*	*E. typhosa*
Halogens:							
Iodine	1:650	1:3520	1:3370	0.2	0.2	32.0	18.1
Azochloramid	1:7800	1:26,000	1:101,000	0.3	0.08	236.3	543.0
Phenols:							
Hexylresorcinol	1:1450	1:1620	1:1770	0.9	0.8	14.7	9.5
P-hydroxyphenyl-n-amyl sulfide	1:2800	1:2700	1:2700	1.0	1.0	24.5	14.5
O-n-hexylphenol	1:3125	1:2900	1:2800	1.1	1.1	26.3	15.0
P-hydroxydiphenyl sulfide	1:3125	1:2500	1:3125	1.3	1.0	22.7	16.8
Phenol	1:224	1:110	1:186	2.0	1.2		
Mercurials:							
Metaphen	1:2070	1:1370	1:5665	1.5	0.4	12.5	30.5
Mercurochrome	1:90	1:12½*	1:140	7.2+	0.6	0.11−	0.75
Merthiolate	1:4220	1:25*	1:2660	169+	1.6	0.23−	14.3
Silver compounds:							
Silver lactate	1:100	1:110	1:690	0.9	0.15	1.0	3.7
Silver citrate	1:610	1:600	1:4500	1.0	0.14	5.5	24.2
Silver nitrate	1:140	1:80	1:1250	1.8	0.11	0.7	6.7
Silver protein strong, U.S.P.	1:25	1:15	1:175	1.7	0.14	0.14	0.94
Silver protein mild, U.S.P.	1:30	1:12*	1:70	2.5+	0.43	0.11	0.38

* Failed to kill. A more concentrated solution could not be prepared.
All results are the average of four determinations.

toxicity with high bactericidal potency against *Staphylococcus aureus* (gram +) and *Eberthella typhosa* (gram −). They are followed by Hexylresorcinol and the phenolic compounds. Both groups are not only very efficient germicides but also comparatively free from any bacteriostatic effect.

The silver compounds, with the exception of silver protein mild, U.S.P., scored very favorably being more efficient against *E. typhosa* (gram −) than *S. aureus* (gram +). Their solutions quickly deteriorate, however, which means that they must be freshly prepared. This is a decided disadvantage.

Of the newer organic preparations Azochloramid, Hexylresorcinol and Metaphen scored the highest. The first two do not exhibit any appreciable bacteriostatic action while Metaphen showed a pronounced effect. Mercurochrome and Merthiolate cannot be relied upon to destroy *S. aureus* by the above technique although they rated considerably better against *E. typhosa*.

This method aims to measure the relative efficiency of germicides which are to be used to kill bacteria in the presence of living tissue. While it is not claimed that actual conditions are duplicated, it is believed that the procedure approaches nearer an "ideal" *in vitro* method than any which has been reported. The organic matter is a mixture of serum and embryonic extract, a medium somewhat analogous to the tissue-fluid found *in vivo*. The correct proportion of organic matter cannot be known since it varies greatly with different infections and conditions. The ten-minute period of exposure is probably the maximal time for effective germicidal action on a cut surface or mucous membrane.

This does not mean that a germicide with a low toxicity-index will be harmless to living tissue cells at any concentration. Due consideration should be given to the killing dilutions of the germicides, in the presence of organic matter, on both tissue and bacteria. The germicides, when actually used, should be diluted to the proper strengths. It may be seen from the results that a 1:100 dilution of iodine would be more toxic to tissue than a 1:100 dilution of mercurochrome. However, if a 1:1,000 solution is used, the iodine would be harmless to tissue but still bactericidal, whereas mercurochrome, though not harmful to tissue, would exert no bactericidal effect.

SUMMARY

1. An improved method is proposed for the evaluation of the efficiency of germicides intended for clinical administration.

2. The compounds are tested for their toxicity to living embryonic tissue as well as for their ability to kill bacteria.

3. A number known as the toxicity index is determined which is defined as the ratio of the highest dilution of disinfectant showing no growth of embryonic tissue in ten minutes to the highest dilution required to kill the test organism in the same period of time.

4. The halogen and phenolic compounds (iodine, Azochloramid, Hexylresorcinol, etc.), appear to be superior to any of those tested by the above technique. They are also comparatively free from any bacteriostatic action.

Of the newer organic compounds Azochloramid, Hexylresorcinol and Metaphen rate the highest. Metaphen shows a very pronounced bacteriostatic effect.

Merthiolate and Mercurochrome cannot be relied upon to kill *Staphylococcus aureus* (gram +) by the above procedure although they rated considerably better against *Eberthella typhosa* (gram −).

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A NEW BACTERIAL SPECIES ISOLATED FROM THE CHUCKAWALLA (SAUROMALUS VARIUS)[1]

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Received for publication January 10, 1939

Twenty-five chuckawallas (*Sauromalus varius*), originating on the San Estaban Island, in the Gulf of California, were received by the San Diego Zoological Society on April 7, 1937. Tumor-like growths in the inferior cervical region were noted in the majority of these specimens. Many of these tumor-like growths were comparable in size to the head of the reptile (fig. 1).

The growth *in situ* resembled an interstitial tumor being surrounded by a strong fibrous capsule connected to the surrounding tissue only by a fibro-vascular neck. A study of the ablated tumor reveals a fibrous wall lined by a layer of tissue consisting of masses of flattened or somewhat rounded epithelial cells separated by bands of fibrous stroma. The capsule is definitely vascular and heavily infiltrated by mononuclear cells.

Within this vascular capsule are concentric layers of a homogeneous amyloidal substance, in which are embedded desquamated cells resembling those in the viable tissue of the capsule. The center of this mass consists of a small caseous accumulation of cell detritus and bacteria.

The most conspicuous tumor-like masses are to be found in the inferior cervical region, although one large tumor was found in the leg (fig. 2). All of the chuckawallas we have studied, living or dead, have shown numerous small tumors, measuring from 1 mm. to 2 cm. in the buccal cavity. The majority of these small tumors are found on the floor of the mouth and at the base

[1] Assistance in the preparation of these materials was furnished by the personnel of Works Progress Administration Official Project No. 8286-D.

of the tongue and resemble pearl epitheliomas. The process of development of the mass is slow.

Bacteriological studies of the tumors revealed a definite organism in pure culture. This organism has been isolated in pure culture from tumors in all chuckawallas of this species studied. Soon after we began our studies on this organism, F. Duran-Reynals and H. J. Clausen (1937), reported "A contagious tumor-like condition in the lizard (*Anolis equestris*) as induced by a new

FIG. 1

bacterial species, *Serratia anolium* (Sp. N.)." At once, a similarity between the conditions and the organism was apparent. Dr. Duran-Reynals kindly sent us a culture for comparison. A comparative study showed the organisms to be, though similar in many respects, different species.

Morphological appearance. The typical form of our organism is a small rod, 1½ to 3-microns in length and 0.2 to 0.6 microns in breadth; axis straight; sides parallel; ends rounded; arranged in clusters or palisades. There is a distinct pleomorphism in older

cultures, a cocco-bacillary form predominating. The organism is motile by means of three to six peritrichous flagella (fig. 3).

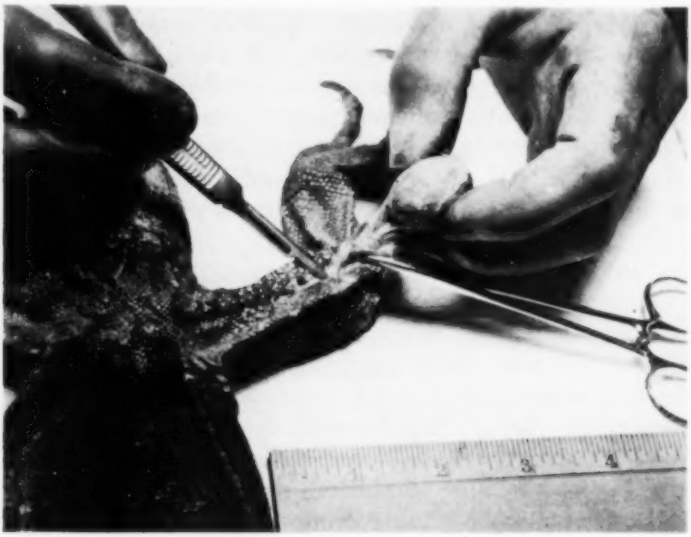

FIG. 2

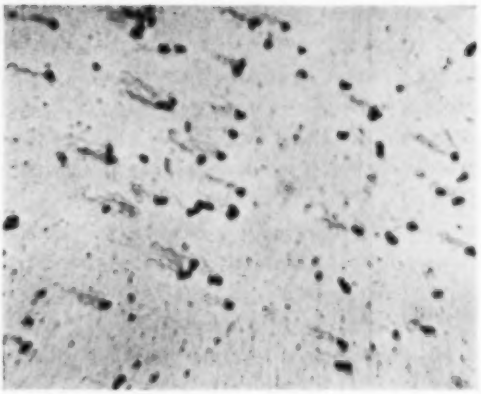

FIG. 3

While the organism in some freshly isolated cultures seems to be surrounded by a capsular material, this has not been definitely

demonstrated. The organism is gram-negative, non-acid-fast; while in older cultures a bipolar staining with methylene blue is evident.

Surface colonies on agar plate. After 24 hours incubation at 37°C. isolated colonies are typically circular to slightly irregular in shape varying from 1 mm. to 3 mm. in diameter; low convex; amorphous, smooth, glistening, entire to undulate, translucent, butyrous, and easily emulsified. There is a marked spreading in young cultures with the smooth glistening colony predominating, although on older cultures the colony may show a radial structure and rough type of colony.

Growth on agar slope. After 24 hours at 37°C., along line of stroke growth is abundant, spreading, slightly raised, smooth, glistening, undulate, butyrous and easily emulsified. Later changes are similar to those occurring on agar plate. A definite slightly pungent odor is noted.

Growth on gelatin. Rapid growth is best at surface. Liquefaction is rapid, infundibuliform on the first day and complete in 3 days at 37°C. A blackish flocculent sediment is formed.

Growth in fluid medium. Broth 24 hours at 37°C., initial growth rapid with moderate uniform turbidity. There is a slight flocculent deposit which disintegrates incompletely on shaking. The faint ring growth around wall of tube breaks away on shaking.

Peptone water. 48 hours at 37°C., same as for broth except for a blackish flocculent sediment which is difficult to break up.

Hydrolized casein. 48 hours at 37°C., same as for broth except for a faint fluorescent yellowish-green pigment diffused throughout the medium.

Growth on rabbit-blood agar plate. Abundant growth; broad zone of alpha haemolysis in 24 hours; total, in 48 hours at 37°C.

Loeffler's serum. Growth raised; digestion rapid, complete in 6 days at 37°C. Medium browned.

Growth on glycerol potato. Abundant raised butyrous growth showing a faint yellowish-green pigmentation diffuse throughout the medium in 2 or 3 days at 37°C. or room temperature.

Metabolic properties and resistance. Aerobic growth only on surface to 1 mm. below surface on glucose agar shake. No growth on deoxygenated semi-solid media. Grows well at 20°C.; growth abundant at 37°C. No growth at 10°C. although cultures remain viable for many months at this temperature. Heating at 56°C. for 20 minutes kills the organisms.

Pigment. The organism produces on certain media a faint but definite yellowish-green fluorescent pigment which gives diffuse coloration to the medium. The production of this pigment was best shown on glycerol potato and hydrolized casein (tryptone and glycerol) to which had been added small amounts of $MgSO_4$ and $K_3(PO_4)_2$. The pigment is water-soluble but is insoluble in alcohol, chloroform, ether, and acetone. There are definite cultural variations in pigment production. Freshly isolated cultures usually produce pigment, while in subcultures the property is subject to discontinuous variation. The ability to produce pigment once lost is not recovered (*in vitro*). A definite yellowish-brown to blackish sediment is always produced in peptone water and gelatin by cultures which produce pigment on other media.

Biochemical reactions. Fermentation of sugars. Acid is produced in glucose, sucrose, mannitol, glycerol, salicin, and maltose. Lactose, xylose, arabinose, galactose and levulose are usually attacked slightly. Dulcitol, dextrin, inulin, starch, rhamnose, and raffinose are seldom if ever attacked. Small amounts of gas may be produced in glucose, sucrose, mannitol, salicin, maltose, galactose, and levulose.

Milk. Coagulated and peptonized. An initial soft curd is formed at pH 5.0. As peptonization proceeds the medium reaches pH 7.2 in 10 days. Purple milk is decolorized but later a clear whey shows an alkaline reaction while the curdled portion remains decolorized.

Indol is not produced.

Methyl-red test positive.

Voges-Proskauer test negative.

Nitrate reduction. Tested in liquid and solid media, nitrates are reduced.

Ammonia is actively produced.

Hydrogen sulphide. In semi-solid PbAc media a faint discoloration is produced.

Catalase is produced.

Diastase is not produced.

Pathogenicity. The organism was pathogenic to certain cold-blooded animals but non-pathogenic to guinea pigs in moderate doses. A soluble toxin was produced.

COMMENT

From the above data there is no doubt that we are dealing with a disease of chronic type in *Sauromalus varius.* The disease is produced or stimulated by a bacterium. This is shown by the consistency with which this bacterium was isolated from lesions of every chuckawalla studied during several consecutive months and also by the production of experimental disease in reptiles by inoculation with the same bacterium. Lesions similar to typical young tumors were produced, from which the causative organism was again isolated. We had no opportunity to experimentally inoculate reptiles of the same species (*S. varius*) as examination of all available specimens in the Zoo displayed these tumor-like processes already in some stage of development. There is no evidence on hand to show that the disease is fatal to the species in nature.

As far as the classification of the organism is concerned, no final conclusion has been reached. In many respects the organism is very like *Serratia anolium* especially in bio-chemical activities; however, morphological differences and the production of a faint but definite greenish-yellow fluorescent pigment which appears to be spectroscopically identical with the fluorescent pigment produced by *Pseudomonas aeruginosa* and *Pseudomonas fluorescens,* would allow one to place the organism in the genus *Pseudomonas.* However, as we have recently received information that the next edition of Bergey's Manual will limit the *Pseudomonas* group to *lophotrichic* organisms we are temporarily placing our organism in the genus *Bacterium* and describing it as *Bacterium sauromali.*

SUMMARY

A new bacterium has been studied which is associated with a chronic disease in *Sauromalus varius*, characterized by tumor-like lesions of a rather slow evolution. The organism is a chromogenic, gram-negative, non-spore-forming, motile rod which has been temporarily classified as *Bacterium sauromali*.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

WINTER MEETING OF THE EASTERN MISSOURI BRANCH

SCHOOL OF MEDICINE, WASHINGTON UNIVERSITY, ST. LOUIS, MO., FEBRUARY 7, 1939.

EPIDEMIC OF NURSERY DIARRHEA. *J. P. Costello*, St. Louis County Hospital, St. John's Hospital; *H. E. Lind*, St. Louis County Health Dept., St. Louis County Hospital.

This report includes a series of 26 infantile diarrhea cases with 11 deaths occurring at the St. Louis County Hospital during the Winter—Spring season of 1938.

From a bacteriological investigation we feel that in this epidemic we were dealing with a pathogenic strain of *Proteus vulgaris* associated with *Escherichia coli*. We feel too that a contributing factor is the absence, or an extremely low content, of free hydrochloric acid which probably favors the growth and production of such bacteria capable of producing a toxic condition as found in these infants. We realize of course, the possibility that this organism *P. vulgaris* and its possible toxins may not have been the primary cause of these diarrheas but may have been secondary.

We feel that our epidemic was similar in many respects to those reported in various hospitals throughout the temperate zone. We feel, too, that the severe toxemia was a result of the absorption of toxins from bacterial development in the gastro-intestinal tract.

We fully realize that *Proteus vulgaris* is found in healthy individuals, but is rarely found predominantly in the intestinal tract and rarely if at all in the stomach, except under pathological conditions.

The disease was characterized by its constantly severe and frequently fatal toxemia. Its onset, cause and duration is so similar in these cases as to strongly suggest that this disease be classified as a communicable disease.

REINFECTION IN POLIOMYELITIS. *Edwin H. Lennette, and Francis B. Gordon*, Department of Pathology, Washington University School of Medicine, and the Department of Bacteriology and Parasitology, University of Chicago.

Eighteen rhesus monkeys recovered from infection with the MV virus strain were inoculated nasally with the same strain; only two of these responded, showing weakness and fever. In the light of the criteria laid down by Sabin and Olitsky, these monkeys represent, at best, questionable cases of reinfection.

Twenty-four MV-recovered monkeys were inoculated nasally with the Philadelphia 1932 strain; 16 showed clinical signs of reinfection, 8 succumbing to the disease.

Neutralization tests prior to administration of the heterologous virus

revealed that antibodies were present against the MV but not against the Philadelphia strain. After infection with the Philadelphia virus, antibodies against this strain appeared in the serum. Immunologic differences between the two strains are described.

THE AGGLUTININ RESPONSE TO BRUCELLERGEN. *T. E. Kircher, Jr.*, Department of Medicine, Washington University, St. Louis, Mo.

Skin tests with Huddleson's Brucellergen were performed on a series of patients who previously had shown no serum agglutinations against *Brucella abortus* strain number 456. Fifty of those having negative skin reactions; i.e. no edema, induration or erythema persistent at 72 hours, were retested for the presence of agglutinins after intervals of from one to eight weeks. Forty of these, or 80 per cent, had a definite response; i.e. showed an agglutination of *B. abortus* No. 456 in titers of 1-40 to 1-640 dilution. There was no similar response to skin tests by either rabbits or guinea pigs.

It is suggested, therefore, that in cases of suspected Brucellosis, agglutination tests should be performed preliminary to skin testing with Brucellergen lest subsequent tests reveal agglutinins to a titer so high that a false diagnosis is encouraged.

CHICK EMBRYO INOCULATION AS A DIAGNOSTIC AID IN VARIOLA AND VARICELLA. *Floyd S. Markham and George S. Bozalis*, Department of Bacteriology, School of Medicine, Washington University, St. Louis, Mo.

During a small outbreak of variola in the winter of 1938-39, atypical cases were frequently observed which were difficult to differentiate from varicella. The technique of chick-embryo inoculation was employed as a diagnostic aid. Strains of variola virus were isolated by direct inoculation of vesicle content onto the chorioallantoic membrane in 5 instances associated with atypical symptoms or lesions. Variola virus was identified by neutralization test performed on the chick membrane with anti-variola or anti-vaccinia rabbit serum.

Vesicle fluids from 5 cases of varicella and 4 of herpes zoster failed to produce infection of the chick membrane.

NOTES ON THE KAHN REACTION WITH ANIMAL SERA. *Wm. H. Gabby*, City Hospital Laboratory, St. Louis, Missouri.

INCLUSION BLENNORRHEA. *L. A. Julianelle*, School of Medicine, Washington University, St. Louis, Missouri.

MOTION PICTURE FILM ON RABIES. *J. J. Bronfenbrenner*, School of Medicine, Washington University, St. Louis, Missouri.

SPRING MEETING OF THE EASTERN MISSOURI BRANCH

SCHOOL OF MEDICINE, WASHINGTON UNIVERSITY, ST. LOUIS, MO., APRIL 11, 1939.

BACTERIOLOGICAL DIAGNOSIS OF GONORRHEA IN THE MALE. *Axel Gronau*, School of Medicine, Washington University, St. Louis, Mo.

A total of 819 bacteriological examinations was made on 221 male patients, of whom 160 were definitely proven to have gonorrhea by bacteriological ex-

aminations as well as by clinical symptoms. Sixty-one patients were assumed to have some genito-urinary infection other than gonorrhea, and while the actual cause of the infection was not established, at no time were gonococci found by the smear or by the cultural method. Each bacteriological examination consisted of smear and culture, and the efficiency of the smear method and the cultural method as a means of diagnosis was compared.

The smears were stained according to Hucker's modified Gram's staining procedure. The culture medium consisted of a 5 per cent rabbit blood chocolate agar. Cultures were incubated at 36°C. for about 40 hours under 10 per cent CO_2 tension. The oxydase technique was employed for the detection of gonococci on the culture.

In 88.16 per cent the findings of smear and culture agreed, and disagreed in 11.84 per cent, taking the 819 examinations as 100 per cent.

The number of total positive findings, where the presence of gonococci was revealed either by smear or culture, or both methods, was 185. Taking this number as 100 per cent efficiency in detecting gonococci, it was found that in 13.52 per cent the culture was able to detect gonococci where the smear definitely failed to do so. In addition in 7.03 per cent the culture revealed gonococci, where the smear was doubtful (±). In 2.70 per cent the smear was positive and the simultaneous culture negative.

An analysis is given of those instances, where the smear was doubtful (±) and the simultaneous culture negative. It was felt that here the deciding factor should be the culture, in the sense, that if no gonococci were found on the culture, the suspected organisms in the smear should be assumed not to be gonococci.

OBSERVATIONS ON CHAPMAN'S SYSTEM FOR DETERMINATION OF STAPHYLOCOCCUS PATHOGENICITY. *MacDonald Fulton, R. O. Meuther and Sister Agnes Gerard Knowles*, St. Louis University School of Medicine and Firmin Desloge Hospital, St. Louis, Mo.

68 of 102 freshly isolated strains of staphylococci were "probably pathogenic" according to Chapman's PHCVBM system of tests. In addition, 16 strains were coagulase positive but negative in one or more of the tests on crystal violet, brom-thymol-blue or mannitol agars. 18 strains which were coagulase-negative were negative in most other tests.

Results on crystal violet and brom-thymol-blue media were not always reproducible. Among the important factors were the age and amount of the inoculum. Reading of brom-thymol-blue agar tests was complicated by strains giving a greenish growth, instead of the usual orange. The hemolysis test would be improved by some agreement as to what constitutes true hemolysis, as distinct from pseudo hemolysis.

Statistically, there is a significant association between ability to liquefy gelatin and ability to coagulate plasma. The gelatin stab culture method is slow and unreliable. A gelatin agar plate tested with ammonium sulfate proved satisfactory.

Statistical examination of some of the available data (Chi square, r by Sheppard's method) indicates a high degree of significance for Chapman's system of assessing pathogenicity when compared to clinical and *in vivo* findings.

NOTES ON THE COAGULASE REACTION OF STAPHYLOCOCCI. *Francis W. DiRocco and MacDonald Fulton*, De-

partment of Bacteriology, St. Louis University School of Medicine, St. Louis, Mo.

A study was made of the effect of various factors on the coagulase reaction. Cultures and plasma (rabbit) could be aged several weeks without altering the result of the test. Eight washings of suspensions from agar slants failed to remove or appreciably decrease the coagulase activity of the cells. Coagulating activity was observed in the washings, suggesting that coagulase, although closely associated with the cells, is an extracellular product. Coagulation was more rapid in cultures grown in broth decalcified with oxalate than in controls. Positive reactions were obtained more rapidly when tests were conducted in shallow layers. At room temperature twice as much time was required to form clots as at 37°C. Room-temperature tests were difficult to read, the clots being threadlike in appearance. Production of coagulase was considerably decreased when organisms were grown in an atmosphere of about 25 per cent CO_2.

ANTISEPTIC VALUE OF CERTAIN PHENOLIC OINTMENTS. *W. C. Clark*, James F. Ballard, Inc., St. Louis, Mo.

The author prepared a series of 2% Phenol Ointments in several fatty bases, he also added certain agents he thought might act as partition agents to carry the phenol away from the fat. Petrolatum; Petrolatum with added fat-like bodies such as; Castor Oil, Cocoanut Oil, Hydrogenated Castor Oil, "Crisco," Soap, Sodium Lauryl-sulfonate, Glycerin, Water, Woolfat, Proteginx, Glyceryl Monostearate and Propylene Monostearate were each made into Phenol-petrolatum Ointments. When these Ointments were tested on 10% serum agar plates using F.D.A. method with *Staphylococcus aureus* A 209 as test organism, not one produced a zone of inhibition!

Phenol, Thymol, Chlorthymol, Parachlor-metaxylenol, Para-amylphenol, Para-phenyl-phenol, and Santophen "7", each were made into Ointments from each of the following fatty bases; Petrolatum, Crisco, Spry, Hydrous and Anhydrous Woolfat, and Vanishing Cream. The phenolic strength was 2% except in the case of Phenol which was boosted to 5%.

Tests indicated that Petrolatum was probably the best fatty base for this group, because the best zones obtained were in the Petrolatum Ointments of (1) Parachlor-metaxylenol, (2) Santophen "7", and (3) Para-amylphenol. Vanishing Cream at first appeared to be an ideal base, but prolonged exposure to warmth caused a gradual recession of the zone until the size was less than that of the Petrolatum Ointment.

It was observed that the addition of any one of the following agents usually increased width of the zone: Alcohol 2%, Sodium Lauryl-sulfonate 2%, Glycerin 2½%, Water 2½%, Soap 2%, Glycerol 2½%, Water 2½%.

Of all the Ointments tested only those made from Parachlor-metaxylenol, Santophen "7" and Para-amylphenol had decided zones when tested on 10% serum agar plates using F.D.A. technique and *S. aureus* for test organism.

The author believes the Phenolic substances like perfumes, are held by the fatty molecule by a sort of absorption which prevents the diffusion from taking place by the ratio of solubilities as it normally should.

THE EFFECT OF 5% SODIUM CHLROIDE ON VARIOUS ORGANISMS; THE EFFECT OF BACTERIOPHAGE ON KREUGERS STAPHYLOCOCCUS AT THIS SALT CON-

CENTRATION. *J. M. Burnett and Helen N. Moore*, School of Medicine, Washington University, St. Louis, Mo.

THE CHORIO-ALLANTOIC MEMBRANE OF THE DEVELOPING CHICK AS A MEDIUM FOR THE CULTIVATION AND HISTOPATHOLOGIC STUDY OF PATHOGENIC FUNGI. *Morris Moore*, Barnard Free Skin and Cancer Hospital, St. Louis, Mo.

THE EFFECT OF 5-PER-CENT SODIUM CHLORIDE ON VARIOUS ORGANISMS. *Helen Norris Moore*, Fouke Fur Co., St. Louis, Mo.

During a course of studies of obligate Halophilic organisms, it was noticed that the growths were always smooth and sometimes quite stringy. Since a smooth variant was always encountered in growths of these organisms in high salt media, it was decided to endeavor to cultivate ordinary bacteria under the same conditions and observe the effect of high osmotic pressures.

A medium containing not less than 5% Sodium Chloride, 5% Gelatin, eggs, soluble starch, beef extract and agar was finally established as the one having the highest content of salt and still able to support good visible growth. The first transplant to the salt medium from ordinary medium will grow in 48 hours and after that 24 is sufficient. The cultures used, together with the number of transplants on the salt medium were as follows:

	No. of Transplants
3 Rough *Eberthella typhosa* (Shwartzman*)	43
All stock cultures of *E. typhosa* on hand at Washington University	12
All stock cultures *Escherichia coli* on hand at Washington University	12
Smooth *E. coli* (fresh isolations)	101
Proteus X-19	76
Shigella dysenteriae (Shiga)	25
Staphylococcus aureus 209	49

The following observations were made:

1. The medium must contain eggs and not less than 5% gelatin in order to obtain good visible growth.

2. The colony appearance of all these organisms on the salt medium is of the normal smooth type. The Shwartzman strains appeared smooth as long as they were cultivated upon the salt medium but reverted at once to their typical morphology as soon as they were transplanted to media of ordinary salt concentrations.

3. The gram-negative bacteria show variations in shape and staining when grown on this salt medium. Elongated, branched and curved cells are common, while in the gram stain the gentian violet is difficult to remove. The cells, however, resume their normal shape and stain when cultivated on ordinary medium.

THE EFFECT OF BACTERIOPHAGE ON STAPHYLOCOCCUS K IN A 5-PER-CENT SALT MEDIUM. *Jack Mehl Burnett;* Department of Bacteriology, Washington University School of Medicine.

Having learned (personal communication) that Moore was able to maintain a variety of organisms on a special medium containing 5 per cent salt without ever observing the appearance of rough variants, we were

* These cultures were supplied by Dr. G. Shwartzman. Their characteristics are described in J. Exp. Med. 1930, **52**, 781–803.

interested to see what effect the presence of salt might have on the susceptibility of these cultures to bacteriophage.

It was found that *Staphylococcus* K would grow slowly in a liquid medium consisting of 5 per cent salt, 5 per cent gelatin, eggs, soluble starch, and meat extract, and would remain susceptible to its homologous phage for at least 8 hours. Despite the fact that this organism rapidly becomes rough on this medium after 8 hours, it should not be considered as contradictory to the observations of Moore for the organism very frequently becomes rough on ordinary meat-extract media.

This medium by itself has no deleterious effect on phage, so that the titre of phage which has been incorporated in this medium is not altered in any manner.

If this medium is inoculated with approximately 1×10^7 organisms per ml. along with about 5×10^4 units of homologous phage per ml., and incubated at 37°C., there is observed a marked growth stimulation in comparison with that obtained in the same medium without the presence of phage. This stimulation was first evident at the end of the first hour, reached its maximum in about 2½ hours, and disappeared with the beginning of lysis after some 4½ hours. The maximum stimulation observed was about 100 per cent over the phage-free control. There apparently is no active phage production during the first two hours and then the phage titre increases about tenfold by the sixth hour. All bacterial counts were made on 2 per cent nutrient agar plates of normal salt concentration, and phage titrations were carried out by the dilution method, using five tubes of each dilution.

This stimulatory effect was not seen when 5-per-cent gelatin was added to nutrient broth of normal salt concentration and the same experiment was carried out.

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The Subscription price for both volumes will be $10.00. Price of single volume (January–June or July–December) $5.00 per volume. For countries outside the Postal Union, add 50 cents a volume. The subscriptions of members of the Society of American Bacteriologists are included in the membership dues and are handled by the Association's Treasurer, Dr. I. L. Baldwin, University of Wisconsin, Madison, Wisconsin. All other subscriptions should be sent to the publishers.

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